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Should the Atlantic be widened?

Mr Alexander Haig's replacement as United States Secretary of State is not in itself remarkable; his successor's task is to head off the growing estrangement of the United States from Western Europe.

The departure of Mr Alexander Haig as United States Secretary of State will not be widely mourned; his vanity and impulsiveness ensured that even those governments elsewhere whose causes he supported were uneasy about his essays in diplomacy. But Mr Haig could hardly have chosen a more momentous time to resign. This week has seen the beginning of the Geneva talks on strategic arms between the United States and the Soviet Union, perhaps the most cheerful development on the international scene for a decade. But the meeting of the heads of governments of the European Community in Brussels this week will also have served to emphasize that relations between the United States and Western Europe are fast running towards the rocks. If Mr George P. Schultz, Mr Haig's successor, can find the time from what is bound to be his immediate preoccupation with the Middle East, he will put Western Europe at the top of his pile of problems. And he will conclude that there are two important conditions that his government must satisfy — that it must acquire a better understanding of what the European problem is and a literally more coherent way of dealing with it.

There are three main groups of issues to be ironed out — one military, one economic and one a mixture of economics and politics. The first, difficult enough, is the simplest. The United States resents what it considers to be the underspending by European governments on conventional forces and the unwillingness of some of them to provide hospitality for the intermediate-range nuclear missiles due to be installed from the end of 1983. The first complaint has substance and should be met, preferably by better planning of the mutual defence of Western Europe. The second complaint, understandable enough, overlooks the delicacy of the domestic politics of those Western European governments, perhaps all of them, dependent to some extent for their survival on voters who are unwilling to give house-room to other people's missiles without something in return. The United States is right to ask that those in Western Europe whom it is willing to help defend should share some of the risks but wrong to expect a favourable response while it was dragging its own feet on arms control. Mercifully, that stumbling block may now have been removed. Much will depend on what happens in Geneva. But the needless delay in embarking on these talks may already have helped to undermine Chancellor Helmut Schmidt's West German government (which, admittedly, has other than nuclear problems on its plate). The need all along has been for a fuller sense in Western Europe of having been consulted in the planning of mutual defence. Mr Schultz and Mr Caspar Weinberger, the United States Secretary of Defense, should bend their energies to that goal.

The economic problem is much less tractable, derived as it is from the pact between Congress and the Administration that the budget deficit should be allowed to run at \$80,000 million or, probably, even more. The strictly domestic consequences of this development are predictable, but will be delayed. The inflation rate, now dramatically reduced to about 6 per cent a year, will either shoot up again within a year or the flickering promise of economic revival will be extinguished. Meanwhile, the United States is meeting its huge budget deficit by borrowing funds from overseas — the effect of the prevailing high interest rate that underpins the banking system, one of whose consequences is the strength of the dollar against other currencies. What is happening

is the opposite of what happened in the late 1960s, when the Johnson Administration paid for the Vietnam war by printing dollar bills and thus exporting inflation. Now, by sucking in funds from the international banking system, the Reagan Administration is exporting deflation.

The consequences for the economies of Western Europe are already serious and much resented. But why should not the United States manage its financial affairs in the way that suits it best, putting up with the domestic consequences as they fall? For two reasons. First, it is no longer possible to separate international financial relationships from international relations generally. With the best will in the world, states in Western Europe thrown into recession by exported deflation will be less able than otherwise to modernize their armed forces and shoulder a full part in their own defence. But they will also be less easily persuaded to other desired goals if they are struggling to deal with domestic difficulties they believe with reason to be caused by the eccentric economic policies of the United States. And it cuts no ice to say that the US Federal Reserve, whose decisions determine interest rates, is constitutionally autonomous. Everybody knows that. But everybody also knows that the Administration is responsible for the budget deficit that makes the Federal Reserve behave the way it does. Second, and more sinister, there is mounting evidence that the United States is seeking the best of both worlds — the right to export deflation but also the right to avoid the consequences that should follow by restraining international trade. Congress and the Administration have embarked on a host of protectionist projects whose result could be to destroy the international monetary and trade system that has sustained us all these past several decades. Europeans in their present mood will go off on their own. Mr Schultz would be well advised to head them off.

The presenting symptom of the third group of urgent issues is the Soviet pipeline for the sale of natural gas to Western Europe (see *Nature*, 3 June, p.349). Arguments about this project have rumbled on for several months. The declared customers for the pipeline's product (France, West Germany and neutral Switzerland) have agonized about the strategic implications of buying Soviet gas, and have publicly decided that they should sign up. The United States, since the accession of Mr Reagan and the appointment of Mr Haig, has blown hot and cold about the implications. Washington has made no secret of its view that they were unwelcome, but has occasionally said that they must be stopped. Mr Haig appears to have made his way at the Versailles summit last month with his expressions of sympathy for the dilemma of European governments placed in this cleft stick. The Administration's decision on Wednesday last week that United States corporations must instruct their subsidiaries and licensees in Europe (and elsewhere) not to supply advanced equipment for the construction of the pipeline is nevertheless unseemly — and a sufficient cause for Mr Haig's resignation.

It is beyond belief that a government so well provided with lawyers can have perpetrated such a gaffe. Corporations registered as such in the United States do not set up overseas subsidiaries for the fun of it, but (rightly) for self-advantage. At some point in the development of their business, they calculate that the time has come when an autonomous overseas corporation would be a better source of revenue than a force of travelling salesmen living



out of suitcases. Qualitatively, the benefits of separate incorporation vary. Sometimes there is tax to be saved. Sometimes, local incorporation provides access to public purchasing that would otherwise be denied. Whatever the reasons for separation, however, the subsidiary corporation becomes a legal entity in the locality in which it is incorporated, and thus subject to local laws. But what if the local laws do not allow that an overseas subsidiary of a United States corporation should tear up a contract because of some instruction from its head office sent retrospectively at the behest of the State Department? This is what is now being required of those with contracts for the Soviet pipeline. The chief result, of course, will be a rash of law-suits and of compensation payments (ironically in convertible currency of the kind that the Administration wishes to deny to the Soviet Union). Further ahead but longer lasting will be the impression left in the minds of Western European governments (and/or their voters) that the United States has slipped into the habit of behaving as if Washington's writ is unconstrained.

Thus the events of the past few days have cast a pall over Western Europe. For the past several years, it has been customary (but not always easy) to say that the defects of the then-current foreign policy of the United States were the consequences of the division of responsibility between the White House and the State Department that seems at last to have been the indignity that Mr Haig could not stomach. Each time this recurrent crisis has come round, another tranche of transatlantic loyalists has been lost to doubt, but European governments have usually buckled to and have been able to hold the line. Always, in the past thirty years, transatlantic cultural cohesion has proved to be a powerful cement. And in any case there has been no alternative to United States hegemony. But now the wind is changing.

Private funds can matter

The legend that foundations are now too small to exert an influence has been shown to be false.

Private foundations have become financially so much smaller than public sources of research funds that it is customary to disregard them. For what, the argument tends to be, can foundations hope to accomplish with their meagre funds when the more immediate threat is to the survival of institutions, the annual running costs of which tend to outstrip the capital resources of foundations? Fortunately, that gloomy inference is not as valid as simple arithmetic would suggest. The lie is neatly given to this canard by the imaginative grant announced last week by the Wolfson Foundation to the two British centres for research in tropical diseases, the London School of Hygiene and Tropical Medicine and the Liverpool School of Tropical Medicine. The circumstances are worth remarking.

Both institutions are relics of the days of what the British used to call Empire, founded as they were to safeguard overseas servants of the Crown from unfamiliar infections by insect-borne parasites of various kinds. Almost by accident, they became places at which physicians, following the precept that prophylaxis is preferable to cure, sought to work out ways of dealing with tropical diseases at their source. Inevitably, they became prominent among the centres, mostly outside the tropics, at which an understanding of tropical diseases was cultivated and cherished. With the passage of time and the collapse of Empire (or colonialism), they have been increasingly supported by agencies concerned above all that physicians and paramedical people from the tropics should have a sufficient training in the diagnosis and treatment of these infections.

From here on, the tale is muddled. It happens (again by accident) that the London and Liverpool centres are parts of the British university system, and that the British government has recently decreed that new overseas students should pay such outrageous tuition fees that they cannot afford to be educated. So, earlier in this academic year, it seemed as if the tropical medicine schools in London and Liverpool would be in serious trouble, deprived of students and then robbed of funds with

which to pay members of their faculties.

The Wolfson grant to the two schools jointly will in the circumstances have a marvellous effect. At a cost of £3 million over five years ("chicken-feed" will say the public research councils) it should be possible to keep the core of each research faculty in being and to arrange that the research they do is at once more imaginative and more closely coordinated than in the past. There is talk of using genetic manipulation to make parasitic antigens, and of understanding the human genetics of susceptibility to parasitic infection. But the prospectus is open-ended. Plainly, it would have been impossible for any public research council to have made such a grant. Yet the chances are high that it will succeed, and that two distinguished centres of research will remain productive. If, anomalously, it should turn out that research in tropical medicine continues to be centred partly in developed countries such as the United Kingdom and the United States, what harm will be done by that? Last month, the European Parliament gave the European Commission a hard time because it had proposed spending part of its meagre research budget earmarked for development in developed countries. The Commission has a longer purse than the Wolfson Foundation, but is less well advised.

Riding for a fall?

The French minister for science and technology may be about to overreach himself.

M. Jean-Pierre Chevènement, minister of science and technology in the Mitterrand government, seems seriously to have blotted his copy-book. For the best part of a year M. Chevènement's inclinations towards the left of his party have been hidden by his devotion to the cause of social transformation by the beneficent, even lavish, support of research and development, but last week he chose to exceed his brief by speaking in public about the war in Lebanon. Worse still, he gave it as his opinion that President François Mitterrand should invite the leader of the Palestine Liberation Organization, Mr Yassir Arafat, to Paris. For his pains, M. Chevènement has been rebuked in the most humiliating way, by means of a statement issued through the President's press secretary to the effect that the President and his foreign minister, M. Claude Cheysson, have no immediate need of Chevènement's assistance. Even Chevènement's friends must be wondering what he is about, alienating his colleagues in the French government just when the final decisions are being made about the budget for next year.

First, the good news. Such is the commitment of the President and the government as a whole to the cause of science and technology as the harbingers of innovation and thus of the prosperity that will buy social transformation that the immediate consequences of M. Chevènement's little lapse are unlikely to be serious. With Chevènement's *loi* now through the Assembly, (see page 6), the chances are good that next year's budget will be protected. Whether the same can be said of Chevènement himself is another question.

While ambition is inseparable from politics, ambition that shows too openly is, paradoxically, mistrusted. In the past year, M. Chevènement has been admired in the true Latin sense of the word (*admirare* = "to wonder at") for the clarity and certainty of his promises and envied because nobody expects them to be delivered for several years, let alone during the reasonable tenure by a bright young minister of a single office. During the past few months, Chevènement has succeeded as well as he could have hoped in carrying his *loi* through the French Parliament, but not without making enemies. Now there is some anecdotal evidence that his attention has begun to wander. His recent speeches have been repetitive; during the debate on his bill in the Assembly last week, he confined himself to what he called "laconic remarks". M. Chevènement seems to be preparing himself for other pastures. He may find that they are less hospitable than he would like if he continues to alienate the Elysée and the rest of the leadership.

Waste in research services damned

UK laboratories lack incentive to save costs

A damning indictment of peripheral waste in British government research establishments was published earlier this week by Sir Derek Rayner, one of the Prime Minister's favourite businessmen and her special adviser on efficiency in government. The report, based on surveys of 19 establishments and concerned exclusively with services for the support of research, suggests that savings of about a fifth in annual budgets would be possible without jeopardizing quality.

The survey is one of several being carried out of efficiency in different parts of the government's business. It consists of a summary of the several separate reports linked together by Sir Derek's own generalizations. One of his central complaints is that in government establishments the cost of support services, ranging from cleaners and doormen to specialist workshops, is lumped together as a central overhead and not apportioned to separate research projects.

The essential failure, says the report, is that neither the provider nor the user of laboratory support services has clear authority and accountability for judging value for money. Rayner says that research establishments should be reorganized in such a way that identifiable research managers are responsible for the whole cost of their projects.

In passing, the survey has uncovered a variety of memorable sources of waste. One establishment was found to have ten deliveries of internal mail each day while the National Physical Laboratory near London maintained eight vehicles that each made less than one journey a month. At the government's Building Research Establishment, a staff of six storemen handled an average of 10 transactions each a day, while the Royal Signals and Radar Establishment was found to have a stock of 17,500 items of scientific equipment with an average age of 8.7 years.

The Central Veterinary Laboratory is reported to have been breeding rats at a cost of £30 a head when suitable animals could have been bought in for about £2 each. The Royal Aircraft Establishment at Farnborough provides itself with an air taxi service at a cost a third greater than commercial charges.

The report on the coordinated survey complains that the government research establishments hold that outside suppliers should be used only when the establishment is too busy or cannot meet the technical need. This, the report says,

"is wrong and costly".

Establishments also tend to be extravagant of land and buildings. The report says that the National Physical Laboratory could save £635,000 a year by giving up 200,000 square feet of floor space and that the Central Veterinary Laboratory should dispose of 245 acres of land now used for breeding animals uneconomically. The report points out that establishments as at present organized have no incentive to make savings of this kind.

The Rayner report also complains that establishments charge too little for services provided to outside users and so "give away . . . public money". Some establishments provide research reports free of charge when they have a "commercial value", others sell products whose cost of production has been underestimated and others undercharge for services "in order to win contracts".

The cost of the bureaucracy itself seems to be substantial. In four establishments covered by the survey on which the report is based, the cost of checking invoices externally doubled the real cost of all the items purchased. But the gravamen of the report's complaint is that "the individual manager of a scientific project is not aware

of or responsible for the actual costs of the support he consumes". The report recommends that responsibility should be transferred to project managers.

The suggested savings on annual costs amount to 14 per cent of the budget now spent on support services within the 19 laboratories and would be made chiefly by shedding 1,518 support staff, or 19 per cent of the total now employed. The report also recommends the disposal of 270 acres of land, 450,000 square feet of accommodation and 200 vehicles, thus raising £6.65 million.

Ministers responsible for the laboratories concerned have apparently agreed in principle to the report's recommendations. Government departments will be performing similar scrutinies of other laboratories in the hope of finding similar savings elsewhere. The plan is to draw up "action plans" for streamlining the support services of individual laboratories by the end of the year. Much remains to be settled, however, not least the questions of how to shed posts and how to change the jobs of researchers so as to encompass greater management responsibility. The reactions of laboratories and staff unions will be eagerly awaited. **Judy Redfearn**

New prospectus for European lab

Substantial changes in the direction and style of research at the European Molecular Biology Laboratory (EMBL) in Heidelberg were being put to the laboratory's council earlier this week by Dr Lennart Philipson, the director-general chosen by the council to succeed Sir John Kendrew's inaugural seven-year reign.

The plans are the outcome of consultation between Philipson and the staff of EMBL and of an almost total lack of contact between the incoming and the outgoing directors-general — ending in a most unknighly deed by Sir John. There have inevitably been clashes within the scientific advisory committee on the extent to which EMBL should pursue a structural approach to biology.

In essence, Philipson's plans call for a shift in emphasis from structural biology towards cell biology, for concentration on fewer areas of research and for better integration of the costly instrumentation division with the laboratory's biological programme. Philipson also says that EMBL's outstations at Hamburg and Grenoble should grow and become more autonomous, and that the laboratory should increase its role as an international training centre, offering more technical courses and eventually a PhD programme.

Another of Philipson's plans is to replace the system of indefinite tenure for a few of the 250 of his staff with one of rolling tenure for up to a quarter of them.

In practice, this will mean that those with tenure will always be on five years' notice. Philipson believes this to be the best way of retaining flexibility while attracting good scientists for short periods. Philipson says that in part this proposal is a response to an unexpected legacy inherited from Kendrew. As soon as he was named director-general in November 1980, he says, he asked that no additions be made to the four tenured staff without consultation either with himself or with the chairman of this council. Kendrew, however, afterwards endowed eight staff members with tenure, five of them within his last three months, without consultation. He was, it appears, quite within his rights to do so (but when asked earlier this week about his reasons refused to comment).

The intended difference in style of research in the next five years reflects a change in both time and directors-general. Whereas Kendrew encouraged certain biological themes at the laboratory, he was prepared to back individuals whose research was not closely allied to any one of them. Philipson, however, tends to the view that backing individuals was even then an outdated approach, and says that the modern need is for a team approach to major problems in biology. Philipson thus intends to concentrate the biological research of EMBL on membranes and on the process of differentiation. Membrane biology is the one area of research in which

the laboratory has a high reputation. The work, however, has been mostly on mechanisms of transport across membranes, and Philipson plans to supplement this with a structural approach. As a start, he claims to have recruited a specialist Rosenbush from Basle, who has recently obtained some of the first ever crystals of a membrane protein, the structure of which will be worked out at EMBL.

The laboratory has much less of a reputation in differentiation, and Philipson's plans depend upon the recruitment of new staff. There may soon be a major project on the terminal differentiation of blood cells and later another on growth factors, but competition with other laboratories on differentiation in *Drosophila* is all ruled out. Other projects ruled out or resisted during planning include mobility within protein molecules, chromatin structure and protein folding.

Inevitably the emphasis on some areas of research will be at the expense of others. Whereas Kendrew felt it essential to have a foot in the door of neurobiology, Philipson will close the door. But Philipson, like Kendrew, is committed to instrumentation as a key to the success of EMBL, believing, however, that it should be better integrated into the research projects. About half of the laboratory's budget is spent on instrumentation, with the most advanced project that on low temperature electron microscopy designed to minimize damage to specimens.

The instrumentation division has also been essential to the unquestioned success of the synchrotron radiation outpost at DESY in Hamburg, where EMBL staff have been chiefly involved with building equipment for use by external collaborators. Philipson hopes to succeed where Kendrew failed by persuading the council to increase the staff at Hamburg from 17 to 25. He plans a similar increase at its neutron diffraction out station at Grenoble.

These plans are based on Philipson's appraisal that the outstations have done more than any other part of EMBL to justify its existence as a European laboratory able to engage in research that cannot be mounted nationally.

Both Kendrew and Philipson admit that such a description cannot be applied to much that goes on in Heidelberg, but Philipson emphasizes the increased role he intends for EMBL as a unique centre for training in molecular biology. It remains a manifest disappointment for many observers that the programme of research at Heidelberg is still much as it might be in any large well-funded national laboratory. And it could only justifiably be for that reason, monetary considerations apart, that Philipson might fail at the end of this year to get the 10 per cent budget increase needed to bring EMBL up to its full strength.

Peter Newmark

Chemical weapons treaty

Talking again

Washington

The Soviet Union may be willing to accept some provisions for on-site inspections in a treaty banning chemical weapons. The first hint of Soviet movement on this issue — which has been the chief obstacle in US-Soviet negotiations on chemical arms — came in a speech on 15 June by Soviet Foreign Minister Andrei Gromyko to the United Nations special session on disarmament.

The United States broke off negotiations in 1980 on a treaty that would ban not only the use of chemical weapons, which is already prohibited by international treaty (the Geneva Protocol of 1925), but also their development, production or stockpiling. Soviet refusal to accept any on-site inspections and the Soviet invasion of Afghanistan were cited at the time as the reasons for suspending the talks.

In his speech, Gromyko said that a chemical arms treaty should provide for "a

possibility of carrying out systematic international on-site inspections", of the destruction of existing weapons and of the continued limited production of toxic chemicals that would be permitted for defensive research purposes under a treaty.

The US State Department is officially saying only that it is studying the proposal and that it is too early to comment. The State Department is apparently wary of showing any favourable response until it can assess the substance of Gromyko's statement. The Soviets may elaborate on their proposal at the international disarmament conference which convenes on 20 July in Geneva.

A State Department official did say, however, that the Soviet proposal appears to address at least two of the three concerns the United States has been pressing — inspection of stockpile destruction and inspection of the permitted research production. The third area is inspection of the shut-down and elimination of existing chemical arms facilities.

James Leonard, who was the US representative at the Geneva disarmament

Industrial secrets still in demand

Washington

The arrest of 18 Japanese businessmen in the United States last week on charges of conspiracy to steal confidential computer information from International Business Machines Corporation may really have been just the latest instalment in a long tradition of international technical espionage. According to Professor Alfred Gollin, a historian at the University of California at Santa Barbara, it now appears that at least two self-appointed spies kept tabs on Wilbur and Orville Wright and reported to the British military.

One was C. S. Rolls (of the automobile

first powered flight, and apparently became quite friendly with them. Professor Gollin found that, for a private citizen, Alexander did have unusual entrée into government circles. This included a close working relationship with the secret Balloon School and with a key figure in the British army's aeronautical programme.

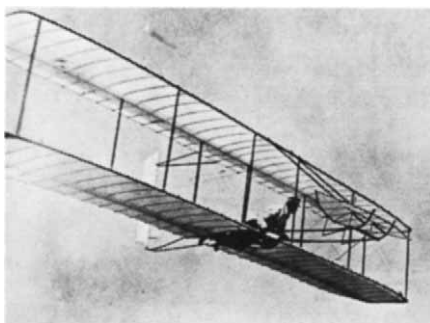
But Alexander also demonstrated the pitfalls of leaving the job to amateurs. He was actually invited by the Wrights to Kitty Hawk to witness their first flight on 17 December 1903, but went instead to the St Louis Exhibition — this was when world's fairs were still worth going to, no doubt.

The Wrights later became convinced that Alexander was in fact a spy. But by that time, the Wrights were embroiled in a patent fight and, according to Dr Tom Crouch of the National Air and Space Museum in Washington, "they thought everyone was spying on them".

In fact, says Dr Crouch, "they developed a conspiratorial mentality themselves", going so far as to send their younger brother, Lorin Wright, to spy on Glenn Curtis, their rival. In what became another amateur performance, Lorin simply marched into Curtis's factory and began taking pictures until he was discovered and had his film forcibly and prematurely exposed by a Curtis employee.

Dr Crouch suggests that any spying that did go on was motivated more by the commercial interests of individuals than the military interests of governments. On the other hand, Edwardian England clearly did have its worries about the Wrights' invention. "The story is not that man can fly", said a British newspaper publisher at the time, "but that Britain is no longer an island".

Stephen Budiansky



company) who in 1908 wrote to the British Committee of Imperial Defence offering to go to France and "draw out" the Wright Brothers. Rolls also bought a Wright biplane, which he offered to put at the disposal of the government. For several years before, the Wrights had negotiated with the British on a sale of their planes, but the deals repeatedly fell through.

The other unofficial spy was Patrick Alexander, an active member of the Royal Aeronautical Society. He first visited the Wrights in 1902, a full year before their

conference from 1969 to 1971 and who is now with a pro-arms control group called the Committee for National Security, says that although the Soviets are clearly making political hay out of the proposal, it is nonetheless more than just words if experience is any guide. "They know that if they make a statement like this, they will eventually be called on it", he says.

Stephen Budiansky

French universities

More change

Paris

The French universities are to be reformed — again. A couple of years ago, the then President Giscard d'Estaing's minister of universities, Mme Alice Saunier-Seïté, set about the universities with a hatchet, amputating this and that to academic screams of "Fascist!" and worse. But now, more quietly and more carefully, the present socialist government of France is setting about a similar task. According to present analysis, Mme Saunier-Seïté was right about many things — it was only her draconian methods that were wrong.

The socialist idea is to base change on a 130-point questionnaire sent out a few months ago to presidents of universities, engineering schools and other such establishments by M. Claude Jeantet, cabinet member at the ministry of national education. The questionnaire was very far-reaching: it encouraged respondents to redefine the role of higher education in France. Jeantet has just analysed the results of that questionnaire — and he will use them to prepare the text of a new law on French higher education for his minister, M. Alain Savary. The new law is promised for the autumn. Although Jeantet will not publish the figures until then, it is already emerging that the government's task will be aided by a remarkable and sudden change in social outlook in the universities — one that has surprised even Jeantet. Now, university staff, often regarded in France as left-wing, see the government and even industry as partners, rather than enemies as appeared to be the case under Giscard d'Estaing. Now, it seems from the questionnaire, the universities are ready to forge links with industry and to run more courses aimed at training students for productive employment — an area in which they have previously been weak. It seems that one remnant of the 1968 university rebellion — a horror of the market-place, and of all government control — is being replaced by a nationalistic commitment to help France escape from the present economic crisis. According to Alain Geismar, one of the revolutionaries of 1968 and now vice-president of the University of Paris VII, the universities are now preparing to consider the provision of continuing education, post-retirement courses and science shops on the Netherlands model.

Argonne prepares for change

Washington

Argonne National Laboratory is renouncing a 16-year old agreement with a consortium of some 30 universities that has governed the laboratory ever since its principal machine, the Zero Gradient Synchrotron (ZGS), was built.

The laboratory outside Chicago is one of several, large, multipurpose energy laboratories whose fate is being deliberated by the White House and the Energy Research Advisory Board of the Department of Energy (DoE) (see *Nature* 6 May, p.3). Rumours that Argonne was to be closed helped prompt the move to change and streamline the laboratory's management. The ZGS itself was turned off a few years ago, leaving the laboratory to carry out other energy studies and nuclear physics, as well as some biology and other basic research.

Back in the heyday of building machines for high-energy physics, many colleges in the middle of the country wanted to have a big machine. When it was decided to put it near Chicago, a compromise was effected, in which other universities in the region could make input, and help get their scientists onto the machine, through a management group, the Argonne Universities Association (AUA). AUA's main job was

to coordinate the high-energy physics programme at Argonne, but it also had management responsibility in other areas. The University of Chicago was also involved in management, through a tripartite agreement involving the university, AUA and DoE, which provides 90 per cent of the laboratory's budget of \$200 million.

Without the ZPG, however, there is no need for regional high-energy physics co-ordination at Argonne. As one scientist formerly at the laboratory says, the AUA structure is also potentially divisive, allowing almost anyone who wants to do something to Argonne to get into the act.

So Argonne's management will gradually be streamlined. AUA will bow out and the University of Chicago will become the manager. Regional universities can still participate through an advisory steering committee. DoE will continue to fund the laboratory.

The laboratory seems unlikely to go out of business, according to DoE, but it may be substantially redirected. For instance, Senator Charles Percy wants to bolster the laboratory's flagging mission by transferring civilian research activities now at Los Alamos and Livermore laboratories to Argonne.

Deborah Shapley

Behind a broad willingness to change in the universities, however, a number of difficult issues lurk. For example, one of the hottest and perhaps most important for French science is the reform of the French PhD system, presently a two-step exercise which is both weaker and stronger than an American PhD. The weak step is the more contentious: the troisième cycle (essentially a two- or three-year MSc course). A good result in the troisième cycle is enough to get a graduate an effectively tenured post as a researcher (assistant at a university, or attaché in a government laboratory). The second step — the major thesis, leading to a doctorat d'état — can take seven years.

Science minister Jean-Pierre Chevènement's law for research and technology, in its present form, contains a commitment to change the system, but no details about how it is to be done; that is up to Jeantet and Savary.

Tenure is, however, a very closely regarded right in France, and the unions, whose influence over the government is strong, will defend it vehemently. One University of Paris professor, whose left-wing leanings combined with a desire to see France do good research perhaps make him typical, said last week that he was "schizophrenic" over the issue. The French system leaves a large pool of relatively untalented researchers at work in France, half of whom would not be in research if they were in the United States.

Robert Walgate

Franco-Soviet space-flight

Up and away

The space-flight of the Frenchman Jean-Loup Chrétien, the first West European in orbit, has been hailed as a triumph for détente and cooperation in both France and the Soviet Union. Although events in Poland had moved a number of French scientists to sign petitions advocating withdrawal from the flight, the Centre National d'Etudes Spatiales (CNES) says that Chrétien's is merely the logical next step in a cooperation programme that has been flourishing for fifteen years.

The coincidence of Chrétien's flight and the final test-run of the US shuttle prompted *Le Monde* to draw a contrast between the French approach and the "cold-war" aspects of *Columbia*. (The fact that, in spite of frequent official denials, the Soviets also appear to be working on a shuttle with, presumably, a similar military potential, was overlooked.)

In their run-up to the flight, the French have exercised considerable tact, even coining the word "spacionaute" to avoid using either the American or the Russian terminology. In return, the Soviet side has permitted a degree of openness about the preparations for the flight that has not been seen since the preparations for the Soyuz-Apollo flight in 1975. While some of this

must have been unavoidable, some revelations seem to have been made purely as an exercise in détente. Five days before the flight, for example, Professor Igor Konstantinovich Bazhinov, the deputy chief of flight ballistics, admitted on Moscow radio that Salyut-7 had undergone unexpected orbital drift, and that a special correction had been necessary to ensure the successful docking of the "international" crew — a degree of intimacy with his audience that is unusual by Soviet standards.

The French experimental programme for the flight was, in fact, outlined in the CNES annual reports for 1980, and includes sensory physiology (including the vestibular, visual and kinaesthetic systems) and the effects of soft radiation on the developmental capacities of unicellular and multicellular organisms. The biological experiments are a continuation of previous Franco-Soviet work using unmanned probes.

Little has been said, however, about the type of space station to be visited by the "spacinaute". Although all CNES releases spoke cautiously of a "Salyut" station, without giving it a number, they were illustrated by a schematic diagram of Salyut-6. Only after the launch this spring of Salyut-7 was it stated that Chrétien would pass his historic week in space aboard what the Soviets say is a more advanced and more comfortable space station.

Vera Rich

French science loi

Who will lose?

Paris

The long-awaited French law for science and technology, which guarantees a 17.8 per cent annual real growth in government civil research spending until 1985, is now almost on the statute books. At present, it lies under the harsh light of an inter-house committee of the French Parliament, which is attempting to reconcile the differences between the views of the Senate (which all but overturned the law) and the Assembly (which supported it). Second readings are to take place next week but it appears that the law will sail through much as planned by M. Jean-Pierre Chevènement and his team, even if those most affected will be reading its provisions with a magnifying glass to see exactly what has, and what has not, been left in.

One thing that was left out is causing the more cautious of French scientists to pause for thought. The law is divided into three sections: an introduction, the law proper (which is quite short) and an annexe. The full force of law attaches only to the law proper, so one question has been what is to go into the law, and what into the annexe? In the research ministry version, fundamental science is mentioned only in the annexe — where there is talk of 13 per cent growth (less than the 17.8 per cent

total growth, reflecting the fact that the Chevènement plan mostly concerns technology). Some deputies at the Assembly, briefed by university researchers, pushed for the 13 per cent to be inscribed into the law proper, but the government refused.

Does this mean that basic research is going to be less well protected against the current French financial crisis than technology? Some French scientists fear so. The question is how far should Chevènement's technological imperatives, outlined in seven major investment programmes from space to biotechnology, encroach on and influence the whole of science. It is beginning to look as if they will be very pervasive.

For example, new accounting methods are to be applied to the big government research organizations, such as the Centre National de la Recherche Scientifique (CNRS), which means that they will be controlled from the ministry, programme by programme, rather than by total budget. The organizations will also be given explicit new tasks, such as the application of their research to profitable ends.

Even the small protection given to basic science by the ministry of national education may be being eroded, as the ministry appears to be adopting the same priorities as the ministry of research. (In distributing its research money, which amounts to perhaps a fifth of the total obtained by universities, the ministry of national education recently asked universities to favour groups already supported through the ministry of research and technology.)

Nevertheless, the fears may be misplaced if French research is compared with the situation of research in other countries. If the 13 per cent figure is respected — and ministry of research officials insist that although only in the annexe, the figure has force — French scientists will be doing far better than their foreign colleagues. For the sum includes a 4.5 per cent annual increase in the number of salaries, and salaries amount to more than two-thirds of basic research costs. The result is that next year's true research budget — what a laboratory director will have in his pocket to spend — would be up by more than 25 per cent in real terms, an increase so large that one senior researcher said last week that it would be "frankly a problem" working out how to spend the money.

For the ministry, the next problem will be how to raise the money promised, against a sombre French economic background (although the promised figures are only averages to 1985) and then how to put into effect certain structural changes outlined in the law. Not least of these is the reform of CNRS and related organizations which will be given new statutes allowing them to make profitable links with industry. CNRS will also get new rules for electing its internal review body, the Comité National. These new rules are

themselves contentious. It appears that the Comité will exclude university lecturers not at present or previously associated with the organization, thus, according to some, deepening the rift between the universities and CNRS.

Robert Walgate

Laboratory animal welfare

Congress in sight of compromise

Washington

After months of negotiation between animal welfare groups and representatives of the biomedical research community, a bill that would tighten up standards for the treatment of laboratory animals has reached the House of Representatives Science and Technology Committee.

This compromise proposal (HR 6245) is now the only serious contender among the several animal welfare bills filed with the House. It will be taken up by the committee later this month when the House returns from its Independence Day recess. The bill avoids some of the extreme measures that some animal groups had pressed for, such as setting aside up to 50 per cent of the National Institutes of Health (NIH) funds now going to work involving animals used for research into non-animal substitutes. But it would impose strict requirements on the care of animals used in federally-supported research. Researchers would have to justify any distress caused to a research animal and ensure that pain was minimized (through the use of tranquilizers and anaesthetics, for example). No animal could be used in more than one major operative procedure, except in special circumstances.

The legislation grew out of hearings held last autumn by a House subcommittee in response to considerable public pressure. At that time legislation for the protection of laboratory animals was not serving the interests of anyone very well. Under the present law, the Animal Welfare Act of 1966, the responsibility for enforcement falls on the Department of Agriculture (USDA), which critics say is understaffed and cannot do a proper job.

Last autumn, for instance, USDA inspectors found only minor violations in Dr Edward Taub's laboratory just weeks before he was indicted under Maryland's animal cruelty law for causing pain and suffering to monkeys. Although Dr Taub protested that he was the victim of a public relations stunt by a group called People for Ethical Treatment of Animals, which had infiltrated one of its members into Dr Taub's Institute for Behavioral Research in Silver Spring, Maryland, he was convicted of the charges and also had a \$200,000 NIH grant taken away from him. Dr Taub appealed against the conviction, and his case is now being heard.

In an effort to obtain the widest possible support for new legislation, the subcom-

mittee staff consulted groups such as the American Physiological Society and the Association of American Medical Colleges when drafting the bill, as well as animal welfare groups such as the Humane Society of America. The bill was reported out of the subcommittee on 9 June by a 14-1 vote.

Universities still have two strong objections, however, that may be raised in the full committee and may prevent any action being taken. One is that the standards set by the bill are quite high. For one thing, the new legislation would apply to rats and mice, which were exempted from the Animal Welfare Act. (Farm animals would continue to be exempt under the new bill.) Research facilities would also have to be accredited by an organization such as the American Association for the Accreditation of Laboratory Animal Care (AAALAC), which since 1965 has certified about 400

"HE'S THE TOKEN RAT."



facilities under a voluntary programme. Universities claim that it would cost \$500 million to bring the remaining NIH-supported universities up to the very high AAALAC standards.

This objection may be answered in part by a 10-year phase-in provision, which the subcommittee staff says should allow much of the upgrading (installation of new ventilation equipment, for example) to be done in the course of routine laboratory modernization and repair.

The second objection is that research facilities will have to set up animal studies committees and in particular will have to appoint one member from outside the university who is "primarily responsible for representing community concerns regarding the welfare of animal subjects". The committees would act much as human experimentation review committees do.

Balancing these possible objections, however, is an apparently growing recognition by the research community of the benefits that mandatory regulations will bring in terms of public reassurance — much as the recombinant DNA guidelines provided what one scientist calls an "umbrella of trust". This may be especially important now that the effectiveness of the Animal Welfare Act and the essentially voluntary system have been called into question.

Stephen Budiansky

US science societies

Women's rights boycott shadow

St Louis

Although the Equal Rights Amendment (ERA) to the US Constitution has now failed, many states will continue to be deprived of important scientific conferences for years to come. And it is possible that some societies will continue to boycott the fifteen states that dragged their heels on the amendment.

The amendment would have added to the constitution an interdiction of discrimination on the grounds of sex. By the extended deadline of 30 June, however, only 35 of the required 38 states had ratified the amendment, which thus becomes a dead letter.

The boycott was first organized in 1977 by the National Organization for Women (NOW), the most prominent feminist organization in the United States. Convention promoters in non-ratifying states have admitted they lost millions of dollars' worth of business during the ERA boycott. Convention sites in ratified states and Canada benefited.

How societies will respond to the lapsing of ERA is not yet clear. Some have abandoned the boycott, but others with large numbers of women members or which are otherwise committed to equal rights for women and minorities are ambivalent about giving their convention business to non-ratifying states.

The scientific societies that adopted the NOW boycott, and avoided Missouri, Illinois and some southern states none of which ratified ERA (see box), include the American Association for the Advancement of Science (AAAS), the American Society for Cell Biology, the Society for Neuroscience, the Society for Developmental Biology, the Endocrine Society, the American Astronomical Society and the Biophysical Society. But AAAS stresses that its policy on meetings is independent of NOW's boycott.

No complete list of boycotting societies exists, but if the experience of Professional Associates of St Louis is typical, fewer than half of all scientific organizations supported the boycott. Professional Associates handles convention planning for nine science groups, of which three supported the boycott. Of those three, two decided to drop the boycott in booking meeting sites after 30 June.

NOW itself has not decided whether to keep the boycott going past the deadline, but the question is likely to come up at its board meeting in July. Even if NOW formally ends the boycott, it will affect where organizations hold meetings for several years if only because many societies make their meeting plans years in advance.

AAAS, the Society for Neuroscience

and the American Psychiatric Association are booked only in ratified states until the end of 1986, the Biophysical Society until 1985 and the American Society for Cell Biology until 1984. The boycott excluded some of the most popular convention sites in the United States such as Atlanta, Chicago, New Orleans, St Louis, Kansas City and Las Vegas.

Some other societies did not honour the boycott only because it was logistically too difficult. The Federation of American Societies for Experimental Biology (FASEB), for example, says it would have liked to have supported the boycott but "The federation's meetings are so large — 15,000 to 20,000 people — we require 50-60 simultaneous meeting rooms. Only one or two cities in ratified states can provide that." The FASEB meeting was held this year in New Orleans in Louisiana, which like most other southern states has not ratified ERA. But two of the six member societies of FASEB, the American Society of Biological Chemists and the American Association of Immunologists, do honour the boycott when they meet as separate groups.

AAAS acknowledges that it has been inconvenienced by its stand, especially when it switched its 1979 meeting from Chicago to Houston in order to hold the meeting in a ratified state. "It's always inconvenient to move a meeting in less than a year's time. We had to be in several

Non-ratifying states

The 15 states that did not ratify ERA are: **Alabama, Arizona, Arkansas, Florida, Georgia, Illinois, Louisiana, Mississippi, Missouri, Nevada, North Carolina, Oklahoma, South Carolina, Utah, Virginia.**

different places in Houston." AAAS meetings can draw 4,000 to 8,000 people.

Germal Sanderson, vice-president for sales of the Chicago Convention and Tourism Bureau, acknowledged that cancellation of business meetings had caused "significant losses". He estimated that 23 groups cancelled previously scheduled meetings, costing the city more than \$11 million. The effect of the ERA boycott could last until 1989, he said.

Nobody knows if the boycott helped or hurt the movement to ratify ERA. People representing boycotted convention areas agree that it did not help the ratification effort and may have hurt it.

Jim Hutchinson, director of convention sales for the New Orleans Convention Bureau, says "It was ridiculous to begin with. It inconvenienced a lot of people and not another state has ratified since it started". NOW itself does not know if the boycott swung any legislators' votes its way. "It certainly was effective in terms of dollars lost" says Judy Murphy, NOW press secretary. "But if it helped, it was just one or many things we did."

Karen Freeman

CORRESPONDENCE

Falklands story

SIR — The history given by Asociacion Argentina Para el Progreso de las Ciencias (*Nature* 10 June, p.450) is in error.

The islands were discovered by the English navigator John Davis, inventor of the backstaff and double quadrant, in 1592. Hawkins was there in 1594.

Commodore Byron took the islands for England in 1765 on the basis of this prior discovery and when the naval garrison was withdrawn in 1774 it left a plaque claiming full sovereignty and allowing Spain to maintain its settlement until 1806.

In 1816 the new United Province of Rio de la Plata having overthrown the Spanish yoke claimed to succeed Spain and a local governor was installed by them in the Falklands in 1828. In 1833 Britain expelled the Argentine soldiers and again raised the Union Jack in the Falklands.

The British prime minister said in 1834 that the British were not prepared to permit any other state to exercise a right as derived from Spain that Britain had denied to Spain herself.

The present conflict does not have its origin in Grytviken, but in the recent naked aggression by Argentina. What was said in 1834 by the British prime minister still stands.

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Burning question

SIR — Most cosmologists feel that the evidence currently available suggests that the Universe originated with a big bang and is now in an expansion phase. One of the more important questions presented by such a scenario is whether or not the Universe will recontract to its primordial super-massive germ. The physical evidence required for resolution of this issue is not yet available.

There is, however, a source which provides the answer clearly and unequivocally. In II Peter 3; 10 it is stated: "But the day of the Lord will come as a thief in the night; in the which the heavens shall pass away with a great noise, and the elements shall melt with fervent heat, the earth also and the works that are therein shall be burned up."

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Whence high IQs?

SIR — In *Nature* of 20 May, A.M. Anderson in his comments¹ on the article in the same issue by R. Lynn entitled "IQ in Japan and the United States shows a growing disparity"², refers to the increase in educational opportunities in Japan since the 1930s, perhaps implying that this increase might be partly responsible for "the Japanese IQ increase", even though Lynn argues against it. While agreeing with Anderson that no intelligence test can be totally culture-free and, therefore, the performance in any such test cannot be unreservedly taken as a true measure of a person's intelligence³, I suggest that what has been found in Japan may indeed be true of all countries where a large proportion of the population has been deprived educationally and socially, over many generations and where people have

overcome this deprivation through rapid democratization of education.

Where opportunities for education and access to knowledge and information, have been limited, education (or lack of it) tends to run in families, with only an extremely small proportion of either class (the educated or the educationally deprived) changing over to the other class. In India, for example, I estimate that education is a hereditary prerogative of between 12 and 20 million persons (2–3 per cent of the population) representing less than 4 million small families and half-a-million "large" families — large in the sense of Indian "joint" families⁴. Probably, less than 10,000 persons every year (out of a total population of 680 million) enter the educationally privileged class from the under-privileged, changing the complexion of neither class.

For those who are educated, the day-to-day problems of survival are lessened. The survival of the uneducated, on the other hand, depends on their possessing abilities which would allow them to cope with the adverse environment in which they must live. Thus the uneducated of today would, as a result of evolutionary pressures, come to possess more "native" (that is inherited) intelligence, than the educated ones on whom by and large these pressures have not operated.

Since the intelligence tests cannot give a true indication of the native intelligence of a person who has been socially, culturally and economically deprived in early childhood, when the cultural patterns are often irrevocably fixed, the above-mentioned difference between the native intelligence of the long-deprived and the long-privileged would show up only when the two groups have had equal opportunities and a comparable environment to live in from the very beginning. A consequence of such a situation would be an apparent rise in the average intelligence of a population where there has been educational deprivation of a large population for a long period, as such deprivation disappears — as has happened in Japan since the 1930s.

If what I have said above is true, one could make two predictions: (1) what has been found in Japan should be found to be also true of other countries and populations where there has been democratization of education on a large scale in a relatively short time; and (2) the average IQ of those who have moved up from a traditionally educationally deprived state should be higher than of those who have been educationally privileged for generations due to circumstances of birth. We must, in that case also, conclude that in the United States, real democratization of educational and related opportunities has not occurred to the extent to which it has in Japan. The fact that the differences in IQ were found by Lynn even at the age of 6 could be merely an indication of the improved environment in Japan, in which the children were brought up to that age, as a consequence of the true educational status of the parents having improved across the population in the earlier generation(s).

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Sequence software

SIR — In the article "Europe leads on sequences" (*Nature* 15 April, p.596), the problem of the collection of nucleic acid sequences is discussed. While it is a crucial problem, it is not the only one. Once collected, the sequences should be easily and quickly accessible to a large number of users, the mode of interrogation of the data base should be manifold, flexible and selective, and the answers should be immediate. Otherwise, the enormous effort required to set up a sequence library would be partly nullified by its limited use. At present, access to the data by most European users occurs by means of magnetic tapes, requested and sent by mail, containing all the sequences and related information stored in the library. The recipient of the tape then needs to read the entire tape on his own computer before the required information can be extracted. This procedure is time consuming and inefficient.

We have developed, on a computer Digital VAX/VMS 11-780 (Physics Institute, GNCB, Bari), an interactive software system for the retrieval and analysis of nucleic acid sequences. This system, which works in real time, offers a large number of options both for perusing and for analysing the data. No specific computer knowledge is required since the users, connected with remote terminals through telephone lines, are guided throughout their work. The main novel feature of the system is the mechanism of data retrieval, which is accomplished through the use of forty keys. Each key can be used alone or in combination with others. Among the available keys are: sequence code, bibliographic citations (journal, year of publication, personal communication), date of entry into the library, number of base pairs, function and biological source. Among eukaryotes, the selection can be made by kingdom, phylum or division, class, genus and species. An analogous selection is offered for prokaryotes. Viral nucleotide sequences can be retrieved by using the virus name, the genus, the family or the host. Biological groups of particular interest can also be selected, for example protozoa, bacteria, algae, fungi, lichens, higher plants, invertebrates and vertebrates. The outputs can be tailored to specific needs, through a choice of what to print and on which output device.

To test our software, we used sequences from a collection of about five hundred, kindly given to us by Dr Greg Hamm (European Molecular Biology Laboratory, Heidelberg) and by Dr Kurt Stüber (Genetics Institute, Cologne). These data were reorganized in order to make them compatible with our software. In planning the system, we took into consideration the fact that precise requirements and standards for a nucleic acid sequence library do not yet exist. Therefore, we incorporated into the software design provisions for great flexibility in terms of additions, deletions and changes.

This system is available to the scientific community upon request and without cost. We hope that it will become a useful research tool.

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NEWS AND VIEWS

Atmospheric chemists *in flagrante*?

from Philip Campbell

OVER the past decade or so, the problems associated with the chemistry of the troposphere (the lowest 10 km of the atmosphere) have attracted increasing attention. The ecological effects of acidic rain and climatic effects of carbon dioxide have both generated many conferences and much media coverage. It was something of a change — even a relief — to see these topics recede (if only temporarily) within the general chemical perspective of a recent discussion meeting*. The organizers' declared aim was to assess the chemistry of the troposphere, particularly as influenced by biota and by the hydrosphere and lithosphere. Fundamental chemical questions were apparent. For instance, free hydroxyl radicals are now thought to be the most significant oxidizers in the troposphere, yet they can barely be detected using current techniques. With such problems being highlighted, the impression left by the meeting is that atmospheric chemists have been caught *in flagrante ignorantia* by their increasingly industrialized planet.

Apart from their chemical production in the atmosphere itself, tropospheric gases originate from volcanoes, oceans, man and his by-products and from biota and their detritus. Much attention at the meeting was focused on biogenic emissions, which represent virtually the sole source of such important trace constituents as ammonia and methane. Biological processes occur both on land and in the oceans of course, but the former, being arguably less homogeneous, present more problems.

A characteristic problem is: how representative are emission measurements of nitrous oxide from a particular site? That is, how reliably can they be extrapolated towards a global budget? Given the significant dependence of emission rates on crop and soil types, for example, and the influence of fertilizers, the need for comprehensive measurements is obvious — doubly so when temporal variations arise due to temperature dependence. More generally, given that compositions of many important trace species are in the parts per trillion (10^{-12}) by

volume range, there were strong pleas for quality control of measurements and for accompanying background data on ambient UV and visible radiation for example. The latter are important because of the photodependence of important oxidation processes discussed below.

The source regions of the biosphere can be split into the oxic (containing free oxygen) and anoxic. The former include the upper layers of the ocean and of soils. As well as such fully oxidized species as carbon dioxide, oxic regions also produce such relatively reduced species as nitrous and nitric oxides from bacterial processes and ammonia from the decay of animal excreta. The anoxic environment (lower soil regions, and the interiors of animals for example) harbours the production of reduced species such as hydrogen sulphide (by sulphate-reducing bacteria) and methane (primarily by anaerobic bacteria in waterlogged soils and in termites).

As well as the specific uncertainties associated with such emission processes, participants highlighted the problems of modulation and transport in the biosphere. This problem is well illustrated by hydrogen sulphide. Bacterial reduction of continental shelf sediments is thought to result in a billion (10^9) tons of H_2S being produced every year, yet only a few million tons reach the atmosphere. The balance is taken up by oxidation in the upper layers of the oceans or, more to the point, by biological oxidation by sulphur bacteria which, in this manner, consume the gas with extreme efficiency. Similar processes have been observed with nitrous oxide.

As layers of bacteria only fractions of a millimetre thick can have a significant role, participants emphasized the care required when measuring fluxes between environmental 'compartments'. In contrast, rarer volatiles such as carbonyl sulphide and dimethyl sulphide (DMS) slip through the bacterial net. DMS is a very important carrier in the global sulphur cycle, being produced by marine algae and having a strong ocean-atmosphere flux. Investigation into possible continental sources of this and other volatile sulphur compounds was placed high on the conference shopping list.

It is now clear that the tropospheric

chemical fates of virtually all the important trace gases are governed by free hydroxyl radicals. As R. J. Cicerone (National Center for Atmospheric Research, Boulder) remarked, this statement would have been ridiculed a decade or so ago when few believed that electrically neutral free radicals could exist in the troposphere. In 1971, however, H. Levy suggested that excited oxygen atoms produced by the well known UV photolysis of tropospheric ozone would react with water to produce free hydroxyl radicals. These highly reactive species, present in minute (and at present barely detectable) quantities, oxidize important trace species such as methane, carbon monoxide, hydrogen sulphide, ammonia and a host of others.

It is obviously important, therefore, that the temporal and spatial behaviour of tropospheric ozone be well understood. An important source is downward diffusion from the stratosphere. However, hydroxyl radicals also play an important part in determining tropospheric ozone concentrations, because they fill the role of active catalysts both in its production (permitting the oxidation of carbon monoxide by oxygen) and its destruction (where ozone oxidizes carbon monoxide to produce carbon dioxide and oxygen). These processes require reaction sequences which involve NO and NO_2 and produce the intermediate species HO_2 . Thus, it was strongly recommended that the atmospheric distributions of these species and of O_3 and OH be intensively investigated. Such work will require the further development of experimental techniques due to the minute quantities of some of the species concerned. Also essential will be the continuation of laboratory kinetic studies since a knowledge of rate constants is central to atmospheric chemical modelling.

The hydroxyl radicals are one example of the significant role of gas-phase catalysis, where minute quantities of a trace species will significantly affect not only the chemical but also the thermal balance of the atmosphere. Thus, those currently concerned about the effects of chloro-

*The Dahlem Workshop on Atmospheric Chemistry was held in Berlin on 2-7 May 1982.

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fluorocarbons on stratospheric ozone (another example of such catalysis) may broaden their attention downwards as the very complex tropospheric pathways become better understood.

Although the oxidation processes described above are important, others may be equally so and are equally ill defined at present. An important example is that of SO_2 . The concentrations of SO_2 and C_2H_2 observed in the remote troposphere are roughly the same although their emissions from combustion are in the ratio of 100:1. Their gas-phase oxidation rates by hydroxyl radicals are similar, so some other mechanisms for SO_2 removal exist. One of these is wet-phase oxidation.

It was remarked that atmospheric aqueous chemists appear to be at the stage reached by their gas-phase colleagues a decade or so ago, with islands of information in a sea of uncertainty. Although the chemical components in atmospheric water are moderately well known, their reactions are still very uncertain. The main oxidizing agents are thought to be H_2O_2 , O_3 and O_2 , and the best (or least poorly) understood process probably the aqueous oxidation of SO_2 to sulphate, of obvious relevance to acidic rain. Aqueous kinetics are strongly pH dependent and depend also on the presence of catalysts such as manganese ions and graphite. There are unpublished observations that indicate that H_2O_2 may be much more prevalent in rainwater than previously thought. Complications include photochemical effects and the fact that equilibrium may not be reached in raindrops.

The probable importance of transition metals for aqueous catalysis was emphasized together with the uncertainties concerning their chemical form. For instance, organic complexes of transition metals have been observed to drastically enhance oxidation of reduced sulphur compounds, while complexation of reduced iron and manganese inhibits their catalytic abilities. The need for laboratory studies of such problems is urgent, together with field identification of complexes in rainwater, aerosols, and so on.

Other uncertainties in the aqueous phase are associated with gas solubilities. G. Ayers (CSIRO, Mordialloc) reported measurements of ammonia in rainfall that indicated considerably lower solubilities than expected. Whether the difficulties lie with the earlier measurements of the Henry's Law constant or with unknown processes peculiar to rainwater is not known. Given ammonia's importance in providing atmospheric alkalinity, such problems (which may also become apparent for other trace species) would appear to be fundamental.

Although the topic of the conference was chemical, the problems of aqueous chemistry are closely linked with the physics of the water cycle itself (for instance, repeated condensation and

evaporation within clouds). Moreover, the means by which atmospheric constituents are incorporated into raindrops, snow and aerosols require detailed investigation, because they not only affect the chemical transformation of species but also represent an important atmospheric sink in global budgets. As an example, and in a memorable display of technical bravura, W.G.N. Slinn (Battelle-Pacific NW Labs, Richland) demonstrated the cleansing action of rain through its depletion of in-flight insect populations.

Given the wide range of outstanding but clearly identified problems associated with the chemistry of today's atmosphere, it would seem presumptuous to consider tackling the equivalent problems of the past, where only a few species have so far been directly identified (as opposed to inferred). Nevertheless, one group of participants was allotted the task of assessing the records of past atmospheric composition. Ice cores, lake and marine sediments and tree-rings can together provide information over the Quaternary period (the past two million years or so), the tree-ring interpretations being dependent on models of leaf uptake and transfer and so on. Ice-cores from Greenland and

Antarctica offer the most direct record of composition, stretching back 10^5 years or more, but practitioners are the first to acknowledge that considerable work is required before the full potential of this resource is realized. Further back in time, the geological record requires a considerable degree of inference to yield compositional information. Past carbon dioxide concentrations are of obvious importance to the climatologists owing to their attendant greenhouse tendencies, and climatic change is more directly documented in sediments than composition.

This has necessarily been a selective review of a wide-ranging meeting, concentrating, for instance, on the inorganic gaseous constituents of the troposphere. But considerable attention was also given to the sources and fates of organic species (for which data are scarce) and of metalloid compounds [such as $(\text{CH}_3)_4\text{Sn}$]. Another extremely important set of atmospheric constituents is the aerosols, whose impact on the chemical balance of the troposphere is profound, and whose composition, physical characteristics and chemical and physical evolution (nucleation and transport) need extensive investigation. □

A corona around the Milky Way

from Joel N. Bregman

MANY new observations in the UV, X-ray and radio wavelength regions indicate that a multi-component corona of hot and cool gas surrounds the disk of our Galaxy. Unlike the disk itself, which is rich in relatively easily studied neutral and ionized gas and cosmic rays, the components of the surrounding region have proved difficult to investigate. Attempts are now being made to fit the new observations to coronal models in which the activity of the disk gives rise to the corona and the heating and cooling processes naturally cause phase changes in the gas, resulting in a complex corona.

Evidence that clouds of gas exist above the disk first came from observations of the Ca II absorption lines against the optical continua of stars, by Münch and Zirin¹. The optical absorption lines are produced by cool ($<10^3.5\text{ K}$) clouds of gas but Münch and Zirin¹, in studies of stars far above the disk, discovered more absorption lines than could be accounted for by gas clouds in the disk alone. Several recent studies, in which UV spectra of hot, distant stars were obtained with the International Ultraviolet Explorer (IUE), support and greatly extend the earlier optical work. Absorption lines

of Si IV and C IV, commonly seen in these UV spectra, originate in either collisionally ionized gas at 10^5 K or photoionized gas at 10^4 K . It is not yet possible to measure directly the distance to individual clouds, but distances can be calculated by interpreting the absorption line velocities with a model for galactic rotation. The clouds probably lie within 30,000 light yr of the galactic disk²⁻⁴ (while, for comparison, the half-thickness of the disk is only 600 light yr).

X-ray observations reveal that another component of the corona is gas considerably hotter than that responsible for optical or UV absorption lines. The Wisconsin X-ray group find an X-ray background flux (1 keV), some of which is associated with the Galaxy and probably originates above the disk in a flattened corona⁵. Unfortunately, they are unable to distinguish between emission from point sources (stars) and diffuse gas, nor do they directly measure the distance to their emitters. Nevertheless, these observations at least are consistent with a hot diffuse corona ($\sim 3 \times 10^6\text{ K}$, $n \sim 10^{-3}\text{ cm}^{-3}$ if the height of the corona is that suggested from optical studies).

Radio observations complete our current understanding of the corona. Observations of the 21 cm hyperfine transition of neutral hydrogen show many

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cool clouds ($< 10^3\text{K}$) with high velocities⁶. Because these velocities cannot arise from galactic rotation of ordinary disk gas, several investigators have suggested that the clouds reside in the corona and may be falling towards the disk⁷⁻¹⁰. Radio measurements of pulsars show that the pulsed radiation is dispersed by electrons of mean density 0.025 cm^{-3} and scale height greater than 1,500 light yr (ref. 11). Finally, a weak low-frequency radio continuum background¹² that is probably synchrotron emission from cosmic rays interacting with the coronal magnetic field is observed. These observations are consistent with the model if there are significant cosmic ray intensities at distances greater than 3,000 light yr from the disk¹³. The galactic disk thus seems surrounded by a thick corona composed of thermal gas at several temperatures ($< 10^4\text{K}$, $10^4\text{--}10^5\text{K}$, $\geq 10^6\text{K}$) as well as cosmic rays and magnetic fields.

Although no single theory explains the presence of all these components, progress has been made in linking some of the components together. Spitzer¹⁴ pointed out that if million degree gas could escape the disk, it would surround the Galaxy with a corona several tens of thousands of light years thick. This corona would lose thermal energy through radiation in a process such that cooling condensations of gas would grow quickly and form into clouds much cooler ($\leq 10^4\text{K}$) than the rest of the corona⁷⁻¹⁰. These clouds may fall into the disk, merge with the interstellar disk gas and eventually re-emerge as hot gas again, in a cycle that has been termed a 'galactic fountain'. For certain temperatures and densities of the hot coronal gas, the theoretical velocity pattern of the cool clouds roughly reproduces that of the high-velocity clouds of neutral hydrogen⁹. While the interior of these cool clouds is probably neutral, their surface may be photoionized by soft X rays, or by photons from hot stars. The ionized surface layer would be the site of the Si IV and C IV absorption lines seen in the UV spectra. Although this model links together the various phases of the thermal gas, it is not without problems, one being that cosmic rays are not included.

As a first step towards remedying this omission Hall¹⁵ has considered the connection between a hot turbulent

corona, and the propagation of cosmic rays and the structure of the magnetic field. In this model, the cosmic rays occupy approximately the same volume as the hot corona and large-scale turbulence in the hot gas causes magneto-hydrodynamic instabilities which result in small-scale

density fluctuations and magnetic field irregularities in the gas. Consequently, cosmic rays are scattered as they propagate through the corona in such a way that the size of the cosmic ray corona and the diffusion coefficient within it can be accounted for. □

Sons and daughters

from T.H. Clutton-Brock

In some species of invertebrates, the sex ratios of emerging adults can vary from nearly 100 per cent males to nearly 100 per cent females^{1,2}. Although the immediate causes of sex ratio variation are diverse — in some species, sex ratios are controlled by environmental conditions during growth while in haplo-diploid organisms they depend on the proportion of fertilized to unfertilized eggs laid — the evidence that they represent adaptive strategies of reproduction is overwhelming². But can diploid organisms such as birds and mammals, where sex is chromosomally determined, vary the sex ratio of their progeny in an adaptive fashion, too? The traditional view is that they can't³ but several recent studies have challenged this position.

The most sensational of these claims, made by Nancy Burley of the University of Illinois, is that coloured leg rings fitted to the legs of zebra finches not only affected their attractiveness as mates but also influence the sex ratio of the progeny they produce⁴. According to Burley, pairs generally bias the sex ratio of their offspring towards the sex of the more attractive parent; for example, those in which the male wears an 'attractive' ring and the female an 'unattractive' one tend to produce male-biased sex ratios. This arrangement, Burley argues, will increase parental fitness.

Not surprisingly, the publication of these claims evoked a strong response. Many laboratory and field studies of social behaviour in birds use colour rings. How many effects might have been obscured — or produced — by the colour of the rings used? How many population explosions — or crashes — could coloured leg rings have caused? Two critiques of Burley's paper have been recently published in *Science*^{5,6} and although Burley rebuts some of the criticisms in the same issue, the small size of her sample and the absence of any relationship between the reproductive success of males and the sex ratio of their offspring in other bird species^{7,8} indicate a strong need for her experiments to be replicated before their generality is accepted.

What of other cases of variation in secondary (birth or hatching) sex ratios in birds and mammals? In many cases,

suggested effects do not approach statistical significance⁹⁻¹¹. In addition, a proportion of significant trends are likely to be fortuitous: sex ratios have universal interest and it is probable that few biologists with access to breeding data fail to look for consistent trends, publishing only if significant effects are found. Since there is no way of estimating the number of those looking for variation in sex ratios, it is impossible to tell what proportion of significant trends could have arisen by chance. There are certainly many data sets in which no sex ratio bias is evident^{8,11-13}; in particular, the search for genetic variance in the sex ratio among outcrossed vertebrates has been largely unsuccessful¹⁴. Where genetic effects have been implicated, they are typically very small indeed; for example, an analysis of 150,000 births sired by 107 bulls found significant differences in the sex ratio of progeny sired by different fathers but, after correcting for the expected binomial variation, the standard deviation of the proportion of males produced was only 1.5 per cent¹⁵.

Nevertheless, a growing number of studies suggest that secondary sex ratios can and do vary among birds and mammals. Birth sex ratios not uncommonly differ from unity in mammals: female-biased ratios are uncommon while a considerable number of sexually dimorphic mammals, including man, show male-biased birth sex ratios which differ significantly from unity in large samples^{16,17}. Exceptions include two species of lemmings which produce around twice as many daughters as sons, apparently because a proportion of females are of XY karyotype with an X chromosome that causes its bearer to be a female and to produce mainly or exclusively X-bearing eggs¹⁸⁻²⁰.

Secondary sex ratios also vary within species. In laboratory mice maintained on low-fat diets, birth sex ratios fell from 50 per cent male in a control group to 24 per cent among experimental animals²¹, while a group of female white-tailed deer maintained on a low plane of nutrition produced 70 per cent sons compared with

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Table 1 Relationship between fawn sex ratio and the temporal interval between onset of oestrus and insemination in white-tailed deer (from ref. 25)

	Hours in oestrus				Total $\bar{x}$
	13-24	25-36	37-48	49-96	
Male fawns	4	12	25	21	62
Female fawns	24	19	15	5	63
Per cent males	14.3	38.7	62.5	80.8	49.6
No. of does	15	15	21	16	67
Fawns/mother	1.87	2.07	1.90	1.69	1.88

47 per cent among females reared on a high plane of nutrition²². Significant associations between birth sex ratios and the timing of insemination relative to ovulation^{23,24} have been demonstrated in several studies of human data and in a recent experiment with white-tailed deer (see Table 1). More males are born early in the breeding season in some seals²⁶⁻²⁹ as well as in red-winged blackbirds⁸ while this trend is reversed in some other birds³⁰. Two recent studies of baboons and macaques show that socially dominant females produce more daughters than sons while subordinates produce more sons than daughters (Table 2). And in several ungulate species there is evidence that more males are produced at high population density³³ while research on rodents indicates that birth sex ratios vary significantly with the population cycle^{34,35}. In none of these cases is the mechanism responsible for the variation in sex ratio yet known but the effects should not be lightly dismissed on this score. Both male- and female-biased mortality can occur during gestation³⁶⁻⁴³ and the possibility of variation in the sex ratio at conception cannot be excluded (see refs 44-46).

A central question is whether variation in secondary sex ratios is adaptive — as is clearly the case in haplo-diploid organisms.

Table 2 Sex ratios in relation to maternal dominance rank in macaques and baboons

Species	Maternal rank		
	High	Low	
<i>Papio cynocephalus</i> (Wild population: ref.31)			
Females	19	7	
Males	10	15	
Per cent males	29	22	
<i>Macaca mulatta</i> (Captive group: ref. 32, Simpson, unpublished) 1972-1981			
	High	Medium	Low
Females	20	12	13
Males	9	19	20
Per cent males	31	61	61
Pre-1972			
	High	Others	
Females	18	7	
Males	6	15	
Per cent males	25	68	

Trivers and Willard⁴⁷ argued that in species where reproductive success varies more widely among males than females and parental investment influences the success of offspring, females should produce more sons at times when they are in superior body condition (and so can afford to invest heavily in each offspring) and more daughters when in inferior condition, perhaps adjusting the sex ratio of their progeny through sex-differential mortality among their offspring. Their theory was subsequently disputed¹⁰ but has since been shown to be robust^{8,48}, though the evidence cited in support of it is unconvincing¹⁰.

Some cases appear to agree with the predictions of Trivers and Willard's theory. The low proportion of males produced by mice reared on poor diets²¹ fits Trivers and Willard's predictions and so, too, may the female-biased sex ratios produced by early breeding grackles³⁰. Other studies have shown that under circumstances of food shortage male neonates are less likely to survive^{49,50}, though an alternative explanation is that increased mortality is one of the costs of faster growth rates, higher metabolic rates and reduced fat deposition among growing males in dimorphic species⁵¹.

However, there are as many trends that apparently contradict the predictions of Trivers and Willard's theory, including the tendency for female white-tailed deer maintained on a low plane of nutrition to produce male-biased sex ratios²² and for dominant baboons and macaques to produce female-biased ones^{31,32}. These results could still be fitted within the framework of the theory if breeding success proved to vary more widely in females than males. Alternatively, parental investment may sometimes have a greater effect on females than males — for it is the effects of parental investment on variance in the reproductive success among male and female offspring rather than the amount of variance *per se* that will determine which sex a parent should invest in most. A difference of this kind has been invoked by Altmann³¹ to account for the female-biased sex ratios produced by dominant baboons: in this species, as well as in rhesus macaques, the reproductive success of females is related to the rank of the matriline to which they belong and dominant mothers may be able to influence the reproductive success of their daughters more than that of their sons. But it is important to remember that neither variation in lifetime reproductive success nor the effects of parental investment on success have yet been measured in both sexes in any wild vertebrate.

Other functional relationships may also be involved. The tendency for late inseminations to produce male-biased progeny could allow individuals to bias the sex ratio of their offspring towards the rarer sex³⁵ — an effect which has been experimentally demonstrated in guppies⁵². In species where females remain in their

natal area while males disperse, sisters may compete with each other for food and male-biased sex ratios may be favoured⁵³, especially when population density is high. Conversely, where populations are divided into small inbreeding populations, competition between brothers for mates may favour female-biased ratios — which could explain the occurrence of female biases among lemmings⁵⁴. Where males are more likely to die before the termination of parental investment, an excess of males may be produced at birth^{16,55} — an argument originally produced by R. A.

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Fisher to explain the existence of male-biased birth sex ratios in man¹⁶.

Alternatively, sex ratio variation may sometimes be a non-adaptive consequence of an adaptive mechanism. For example, in baboons and macaques, females are likely to remain in the troop where they are born throughout their lives and mothers may be able to increase the reproductive success of their offspring by removing as many potential competitors as possible⁵⁶. In several species, there is evidence that female juveniles are more likely to be attacked or threatened by conspecifics than males^{57,58}, and one study has even suggested that this effect may extend to females pregnant with female fetuses⁴⁰, possibly increasing the likelihood that subordinates will abort if they have conceived daughters.

However, the basis for adaptive explanations of sex ratio variation in vertebrates is weak because we know virtually nothing about the extent to which parental investment affects the

reproductive success of sons and daughters in wild populations or the environmental factors that modify its influence. In addition, the very flexibility of adaptive arguments undermines their credibility since there are few trends to which a plausible, adaptive explanation could not be fitted. Before accepting such arguments, we need to know considerably more about the distributions of the effects they claim to explain. How is maternal rank related to the sex ratio in primates where daughters do not inherit their mother's rank? Is high population density associated with female-biased birth sex ratios in species where daughters disperse and sons remain in their natal area? Do species which show particularly biased birth sex ratios also show especially strong sex differential mortality during early growth? And how is sex ratio variation achieved? Until these questions have been examined, we shall continue to be left with some interesting data, a plethora of stimulating ideas and few firm conclusions.

products involved in positional signalling. If their suggestion is correct, understanding how the products of this locus work will be of fundamental importance. What is their evidence? Briefly, they have obtained a series of mutations in this locus which result in the absence of specific parts of the adult appendages. The mutations fall into several discrete classes which can be arranged in an order such that low-class mutations remove only a few distal structures in each appendage, whereas progressively higher-class mutations remove more and more proximal structures (the highest-class mutations appear to kill the embryo). In addition, the functional genetic units defined by the different classes of mutation are arranged on the chromosome in an order that corresponds directly with their phenotypic hierarchy. On the basis of these results, the authors "presume that the functional units in the *decapentaplegic* complex correspond to a series of structural genes encoding activities in morphogenetic positional signalling".

Because this work may comprise the foundation of a detailed genetic and molecular analysis, it is well to ask how good this presumption is. At its basis lies the assumption that loss of distal structures results from absence of positional signalling along the proximo-distal axis. As Spencer *et al.* note, there is no easy way to assess the validity of this assumption. Consequently, one must bear in mind that there may be other less interesting explanations for the preferential loss of distal portions of adult appendages. For example, distal portions of most imaginal disks may proliferate more rapidly than proximal portions; it is possible that in the absence of the *decapentaplegic* gene(s), rapidly proliferating cells may accumulate a toxic metabolite, or run out of an essential metabolite, thereby leading to cell death and consequent loss of structures.

It may help to compare the phenotypes of *decapentaplegic* mutations with the phenotypes of other mutations thought to be primary defects in the cell patterning mechanism. For example, homeotic mutations, exemplified by mutations of the *bithorax* complex⁵, result in particular parts of the animal developing like other parts (for example, some of the abdominal segments developing like the second thoracic segment). That is, they result in the replacement of one cell pattern by another. In addition, such mutations transform the cell pattern of both the larval and

Decapentaplegic — hopes held out

from Gary Struhl

FRUIT flies are composed of around a million cells arranged in a miraculously complex pattern. Each eye, for example, contains about 700 facets arranged in a crystalline lattice; each facet is itself a precise hexagonal array of specialized receptor, pigment and accessory cells. How such intricate patterns arise is a central problem in developmental biology. In the case of the eye, it was once thought that each facet arose independently as the descendent of a single cell. But this simple lineage model fell by the wayside with the discoveries that facets are not clones¹, and that any two cells in a cluster of neighbouring facets can arise as daughters from the same mother cell². Thus, in the eye, as in most other tissues of the adult epidermis, cell patterns are not dictated by cell ancestry, but rather by interactions between cells. And in order for such interactions to give rise to the coherent cell patterns of organs and appendages, there must be mechanisms for organizing and interpreting spatial information in large populations of cells.

At present, we can only speculate about how cells know where they are in a developing tissue, and how they use this information to decide what to do. Whatever the mechanism, however, its

components must be molecules encoded by genes, and this fact provides one of the few approaches to understanding how cell patterning occurs. In principle, it should be possible to identify the particular genes whose products are responsible for organizing and interpreting spatial information, and, once identified, it should be possible to isolate such genes, purify their products and determine their mode of action at the molecular level.

Unfortunately, there is at least one serious flaw in this golden scenario — how does one identify the relevant genes? There are of the order of 10^4 genes in the *Drosophila* genome, of which only a few are probably integral components of the cell patterning mechanism. At present, the only criterion for finding these few needles buried in the genomic haystack is mutant phenotype. But here is a catch-22: without a clear understanding of the mechanism in question, it is difficult to predict the phenotypes of mutations which eliminate its component parts. So choosing the right genes is a difficult business, depending as much on chance and intuition as on objective arguments. And unfortunately, an error in judgement may go undetected for several years.

In a recent paper³, Spencer *et al.* have described a complex genetic locus in *Drosophila*, *decapentaplegic* (*dpp*; otherwise called *held out*⁴), which they suggest encodes a series of related gene

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adult epidermis in a corresponding fashion. A second example is that of mutations such as *engrailed*⁶ and the segmentation mutations isolated and described by Nusslein-Volhard and Wieschaus⁷ which alter the pattern of all segments in a homologous fashion. These mutations affect all segments of the embryo, and in a few cases all segments of the adult. Moreover, they generally result in deletion of a particular portion of the segment and in mirror image duplication of the remaining portion. Again, as in the case of homeotic mutations, these mutations result in phenotypes which may be described as the replacement of one cell pattern by another. Also, like the homeotic mutations, they alter the cell pattern of the embryo, and hence affect cells which have not undergone extensive proliferation.

Whether mutations which result in the

replacement of one cell pattern by another are more likely to be relevant to the process of positional signalling than mutations which simply eliminate homologous portions of cell pattern is anybody's guess. Similarly, it is difficult to assess whether mutations which alter cell pattern in precise ways in the absence of extensive proliferation are of more interest than mutations which cause the elimination of proliferating cells. In the case of the *decapentaplegic* complex, further descriptions of mutant phenotypes at the cellular level may help to bolster the argument that this gene complex plays a specific part in positional information. But ultimately, the validity of this argument, or lack thereof, may only be apparent after all the hard work is done — when we understand the molecular roles of the *decapentaplegic* gene products. □

although antibodies raised against several KLH-linked synthetic peptides could react with influenza haemagglutinin, they did not react with the previously delineated antigenic sites⁸. Moreover, antibodies raised against the haemagglutinin did not react with the synthetic peptides. These results suggest conformation of the molecule is important.

A key question is whether synthetic peptides can form the basis for a practical vaccine. The results so far obtained have great promise. Peptides with differing specificities might even be coupled to a single carrier protein to give a multivalent vaccine. Problems still remain to be solved, however, some applicable to all vaccine candidates and others more specific to particular vaccines. For foot-and-mouth disease, a synthetic vaccine would have to compete with existing vaccines which are produced by highly developed technology and are very efficient and cheap. Nonetheless, existing vaccines have some problems. First, safety: a number of outbreaks of foot-and-mouth disease have been associated with failures of the inactivation process. The most recent outbreaks in Jersey, and on the Isle of Wight, almost certainly derived from such an accident, as was shown by careful analysis of the molecular characteristics of the virus which showed striking identity between vaccine strains and strains isolated from outbreaks (ref. 9 and see J. Brooksby *News & Views* 293, 431; 1981). Clearly, a synthetic peptide overcomes these problems, but in truth, safety is not a problem if formalin is avoided as an inactivating agent. This chemical has well established limitations for viral inactivation but when acetyl ethyleneimine is substituted there are no problems with inactivation of FMDV. A second consideration is effectiveness. There are several problems for existing vaccines; first, antigenic variability. One of the reasons for suspecting that the two regions on VP1 would be effective immunogens was that they vary in amino acid sequence from strain to strain. New synthetic peptides will be required for each strain but the process of cloning and sequencing the VP1 in order to identify new peptides to synthesize should be simple. Indeed, a number of new peptides might be made ahead of time. Some strains grow poorly in cell culture and are poor vaccine strains for this reason, whilst others are very unstable or are poor strains for less clearly defined reasons. These problems may well be solved by the use of synthetic peptides. Similarly, all foot-and-mouth disease virions are relatively unstable, especially at below pH 5.0 and at ambient temperatures; again synthetic peptides are likely to be superior because they are more stable. The existing vaccines are effective after a single dose when given with an adjuvant, usually absorption to aluminium hydroxide and saponin for cattle and an oily adjuvant for pigs. So far, the vaccines prepared from VP1 produced

Synthetic peptides as the basis for future vaccines

from John Beale

MOST antigenic determinants of proteins are thought to be conformational rather than sequential; they depend upon the three-dimensional folding of the molecule rather than on the linear sequence of amino acids. Jim Bittle and his colleagues provide in this issue of *Nature*, p.30, an example of the importance of the primary amino acid sequence for foot-and-mouth disease virus (FMDV). They synthesized peptides corresponding to several regions of the virus polypeptide VP1 and found that one of them, corresponding to amino acids 141–160, when coupled to keyhole limpet haemocyanin (KLH) and given with an adjuvant, produced neutralizing antibodies to FMDV and protection against challenge in animals. Another peptide, containing amino acids 201–213 of VP1, also produced neutralizing antibodies. This is an exciting result for both theoretical and practical reasons.

The fact that alteration of conformation by denaturation or enzymatic digestion greatly changes antigenicity suggests that most antigenic determinants of proteins are conformational rather than sequential. For foot-and-mouth disease, for example, the viral peptides derived from either infected cells or disrupted virus have lost most of their immunogenicity; genetically engineered VP1 is also of low immunogenicity¹. However, several recent lines of evidence suggest that small synthetic peptides having the sequence of antigenic determinants on a protein will, in fact, react with antibody to the intact native antigen and in some instances will neutralize its biological activity.

Langbeheim, Arnon and Sela², for

example, found that a 20 amino acid peptide corresponding to amino acids 89–108 of the coat protein of MS2 phage, when coupled to a carrier, induced antibodies in animals which neutralized the phage and were as effective as antibody to the intact phage or the coat protein. The neutralization test involved the use of goat antiglobulin which may have produced neutralization without the primary antibody binding to a specific neutralizing site. Peptides representing other regions of the coat protein were ineffective. Similar results come from synthetic peptides corresponding to the antigenic determinant regions of diphtheria toxin³ and the natural cyanogen bromide immunizing fragment of the M protein of type 24 *Streptococcus pyogenes*⁴. Success with hepatitis B virus has also been achieved by several groups, although protection in chimpanzees has not yet been reported (see A. Zuckerman *News & Views* 295, 98; 1982). Attempts to reproduce the native conformation of the antigenic determinant by producing a cyclic peptide⁵ did not yield an antigen clearly superior to the linear peptides⁶.

The situation with influenza virus is more confused. Studies of variants using monoclonal antibodies and X-ray crystallography⁷ have provided very precise information about the amino acid sequences and the antigenic sites, so the effectiveness of synthetic peptides would be confidently predicted. In the event,

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in *Escherichia coli* have proved less effective than existing vaccines. Thus, even after two doses, antibody levels are much lower than after a single dose of conventional whole-virus vaccines. Synthetic peptides are reported by Bittle *et al.* to be far more effective than VP1 but this comparison is a bit unreal for two reasons: first, the VP1 they used was prepared by SDS treatment and polyacrylamide gel electrophoresis which is a denaturing step and may hide the sites in VP1 reacting with anti-peptide sera or with native virions. Also, VP1 is a relatively small protein, whereas the synthetic peptide was coupled to KLH, one of the most immunogenic carrier proteins in the immunologist's repertoire. The results of coupling VP1 produced by genetic engineering to a similar immunogenic carrier would have been a more valid comparison. Perhaps the most encouraging feature, however, was the almost equally good results they obtained from a single dose using aluminium hydroxide compared with Freund's adjuvant. The dose schedule and duration of immunity are important aspects of vaccine development. As noted earlier for foot-and-mouth disease vaccine, because of the difficulty of mustering animals for vaccination, protection is required from a single dose, but two to three doses may be given each year. For many other vaccines a two-dose primary schedule can be followed by booster doses over a period of years. The performance of vaccines based on synthetic peptides in these respects will need study, but there is no reason to suppose they will prove less efficient than existing killed vaccines.

The feasibility and economics of synthetic peptide vaccines are difficult to judge. The situation for foot-and-mouth disease, where there is an existing relatively cheap vaccine, is very different from that of hepatitis B, for example, where the vaccine is very expensive and can be made only on a limited scale. Another key factor will be the degree of purity of the peptides required. Lerner and his colleagues did not make any special efforts to purify the peptides that are produced by the Merrifield solid-phase synthesis. Plainly it will be essential to establish that the effects are due to a peptide truly of the sequence deduced from DNA sequencing. The degree of purity ultimately required will doubtless vary according to the application. It will be necessary to have a synthetic process that consistently produces the same product — no doubt the spur of a practical application will lead to improved methods and reduced

costs. The size of the peptide to be made will be a key factor. Another important element requiring more research is the carrier. In the present experiments with FMDV, KLH was used but it seems certain to be superseded because of supply problems. The addition of a carrier such as KLH negates some of the advantages of a defined synthetic vaccine and problems of hypersensitivity to the carrier may occur when repeated injections are necessary. Clearly there are alternative carriers, such as tetanus toxoid or poly-DL-lysine, and this is an important matter for more research.

Similarly, there will be a need for an adjuvant. Aluminium hydroxide is a clinically acceptable adjuvant for man and it is encouraging that it worked for FMDV. The question of the adjuvant is linked to the requirement for a carrier and the best combination will need to be reassessed. Knowledge about adjuvants is at present a dark area of immunology.

The work of Bittle *et al.* is a significant step along the road to synthetic vaccines. It opens up exciting possibilities for vaccine development which should be vigorously pursued. □

The internal evolution of Venus and the galilean satellites

from Sean C. Solomon

THE solid planets and satellites of this Solar System display a great diversity of surface features. The Moon and Mercury and many of the icy satellites of Jupiter and Saturn have heavily cratered terrains that have been little altered for billions of years. Jupiter's moon Io has been continuously resurfaced over periods of millions of years by vigorous volcanic eruptions fuelled by the dissipation of huge solid-body tides. The Earth has a lithosphere underlying the ocean basins that is recycled by plate tectonics over a period of about 100 million years. Despite their differences, all these bodies have been shaped by common physical processes during their formation and subsequent evolution. A continuing challenge, made evident by the many new results on the internal and surface evolution of planets and satellites presented at the 13th Lunar and Planetary Science Conference*, is to understand how these common processes have produced the observed diversity of Solar System objects.

Particular interest at the conference was focused on Venus and the galilean satellites of Jupiter, all objects of recent scrutiny by US and Soviet spacecraft.

Dramatic new results have come from Venera 13 and 14 which landed on Venus on 1 and 15 March respectively (V.L. Barsukov and Yu. A. Surkov, Vernadsky Institute of Geochemistry and Analytical Chemistry; and see *News and Views* 296, 607; 1982). Most notable are the chemical analyses of compositions of Venus soils, derived by X-ray fluorescence, that indicate similarities to a high-potassium alkali basalt at Venera 13 and a terrestrial oceanic tholeiite at Venera 14. On Earth tholeiites, because they comprise the upper igneous crust of all ocean basins, are the most common volcanic rock type.

Discovery of such basalts on Venus supports the hypothesis that the major-element compositions of the two planets and their respective mechanisms for the formation and eruption of basaltic magma are similar.

Detailed comparisons of Venus and Earth with respect to planetary composition, mechanical properties and tectonic evolution were also presented. The bulk density of Venus, after correcting for the effects of compression, is generally estimated to be about one per cent less than that of the Earth¹. The difference is often explained in terms of a slightly different composition for the two planets^{1,2}. K.A. Goettel (Washington University) suggested that Venus may have a bulk density at standard conditions equal to or greater than that of the Earth. Such a result would hold if internal temperatures are significantly higher for Venus than for Earth at comparable depths and if Venus has a thicker layer of basaltic crust³. The required higher temperatures may occur, however, only if Venus has no analogue to terrestrial plate recycling⁴. If Goettel's suggestion is correct, then cosmochemical models for inner planet formation will require revision.

There is much debate over whether some version of plate tectonics operates on Venus. The issue has broad implications for our understanding of heat transfer and surface evolution on silicate planets. H. Spetzler, I.C. Getting (CIRES, University of Colorado) and H. Mizutani (University of Nagoya) have made a new contribution in a theoretical formulation for brittle failure in silicate planets based on stress corrosion theory and the interaction of growing cracks. Including the effects of

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*The 13th Lunar and Planetary Science Conference was held at the Lyndon B. Johnson Space Center, Houston, Texas on 15–19 March 1982.

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water on the rate of crack growth, they have calculated the effective failure strength as a function of depth in each of the terrestrial planets. For Venus, assuming the partial pressure of H_2O in near-surface rocks to equal that in the lower atmosphere, they calculate a very low value of crustal strength (about 10 MPa) and conclude that plate subduction on Venus would be unlikely.

This author and J.W. Head (Brown University) took a different view. Venus must lose an amount of heat per mass roughly comparable to that known for the Earth⁵. Solid planets and satellites transfer internally generated heat to the surface by plate recycling, as on Earth⁶, lithospheric conduction, as on the Moon, and hotspot volcanism, as on Io. Each has been suggested as the dominant mechanism for lithospheric heat transfer on Venus⁶⁻⁸ and we argued that none can be ruled out given our present knowledge of the venusian surface. In particular, plate recycling cannot be excluded once it is recognized that the characteristics of plate divergence and convergence zones on Venus and Earth will be different because of the different surface temperatures (740K on Venus) and surface abundances of water. Whatever the dominant mechanism of lithospheric heat transfer, however, given a global heat loss per mass comparable to that for the Earth, we must conclude that most of the large-scale topographical features on Venus are young compared with the surface ages of the smaller terrestrial planets.

The galilean satellites, because their surfaces are cold and are composed of unusual materials, display landforms and surface histories that are difficult to understand simply by extrapolation from the known outcomes of processes on the terrestrial planets. Europa, for instance, has a high-albedo icy surface criss-crossed by numerous darker lineations of great length and probably of tectonic origin⁹. T.B. McCord (University of Hawaii) and colleagues reported on a new spectral unit map of Europa derived from Voyager multispectral images. At least four distinguishable units occur; the darker lineations are spectrally similar to other

dark units. E. Schonfeld (Johnson Space Center) proposed that the brown colour of the lineations and the low reflectance of the satellite at 3–4 μm may be due to the presence of organic molecules erupted from Europa's interior during episodes of aqueous volcanism. Interpreting the lineations as lithospheric fractures, P. Helfenstein (Brown University) showed that they may be the result of tidal stresses, although the details of such an explanation are considerably more complicated than in earlier versions¹⁰.

The galilean satellites Ganymede and Callisto are similar in size and bulk density, yet their surfaces differ markedly⁹. Callisto displays a surface that has been little altered since an era of heavy impact bombardment. Ganymede, however, preserves heavily cratered units but also contains younger and more lightly grooved terrain, thought to be of extensional tectonic origin, suggesting the hypothesis that Ganymede has undergone greater global expansion than Callisto since the period of high impact flux. P.Y. Huang (MIT) and this author tested this idea by calculating the lithospheric thermal stress that would accumulate from global thermal evolution, including the effects of subsolidus convection and phase changes in H_2O ice, and concluded that Ganymede and Callisto would have had similar stress histories if their initial conditions were similar. If, however, Ganymede began at higher temperatures because of thermal gradients in the proto-jovian nebula¹¹, the difference in stress histories can account for the different surface characteristics. New estimates of lithospheric thermal gradients versus time in Ganymede and Callisto were reported by Q.R. Passey (Caltech) and E.M. Shoemaker (US Geological Survey) from the distribution of viscously relaxed craters of various diameters and by W.B. McKinnon (University of Arizona) from the widths of concentric ring scarps, interpreted as graben, in impact basin structures. Both studies indicate higher thermal gradients on Ganymede than on Callisto in the period 3 to 4 billion years ago.

The detailed tectonics of grooved terrain formation on Ganymede continue to receive special study. M.T. Zuber (Brown University) estimated the displacement that has occurred between large units of cratered terrain on Ganymede under the assumption that concentric arcs of ring structures in different units were formed by a single event; different locations of the centres of curvature for different units suggest displacements of up to 40 deg arc. M.P. Golombek (Lunar and Planetary Institute) derived a limit of one per cent on the increase in satellite radius associated with extension in the grooved terrain on the assumptions that the grooves are graben and that the dip angles of the bounding normal faults are 60° or greater. This limit agrees with that of McKinnon¹¹ based on the lack of tectonic disruption of Galileo

Regio. S.K. Croft (Lunar and Planetary Institute) proposed that grooved terrain is formed by hydraulic fracturing driven by overpressured water during the final phases of global freezing following early differentiation.

Many of the icy satellites of Saturn show extensional features broadly similar to those of Ganymede¹². S.W. Squyres, R.T. Reynolds, P. Cassen (Ames Research Center) and S.J. Peale (University of California, Santa Barbara) drew particular attention to Enceladus, which shows features similar to the grooves of Ganymede and large areas of low crater density. Resurfacing of such younger areas could occur either by extrusion of fresh material or by complete relaxation of topography due to a high thermal gradient. A new model by D.J. Stevenson (Caltech) for magma migration by crack propagation through the lithosphere, when applied to Enceladus, suggests that volcanism may occur by comparatively infrequent eruptions of large volumes of H_2O-NH_3 fluids, lending support to the volcanic resurfacing hypothesis. Squyres and his colleagues showed that tidal dissipation is an adequate heat source for volcanism on Enceladus as long as a forced eccentricity several times higher than the current value existed continuously or episodically through a substantial part of its history.

As the data from Pioneer, Venera and Voyager missions continue to be analysed, hypotheses for the evolution of Venus and of the outer planet satellites will be refined. To test these hypotheses will require new spacecraft observations. The US Galileo mission to Jupiter will help provide data for the jovian satellites, and the Soviet Union has announced plans for additional landers to be sent to the Venus surface to conduct further chemical analyses and imaging experiments. A major gap in our knowledge of Venus, however, is a global map of the surface at sufficiently fine resolution to distinguish among the proposed hypotheses for the tectonic evolution of the planet. While the proposed Venus Orbital Imaging Radar mission has fallen victim to reductions in the NASA planetary programme budget, a less ambitious and less costly Venus radar mapping mission is currently being considered to fill this gap. Completion of such a mission would provide a major step towards a full understanding of the evolution of Earth-like planets. □

Corrigendum

In the *News and Views* article on 'Cause and treatment of atherosclerosis' by C.T. Dollery (*Nature* 297, 286; 1982), the sentence "C.T. Dollery (Royal Postgraduate Medical School, London) described a new method for measuring plasma 6-oxo-PGF, the hydrolysis product of PGI_2 , which uses gas chromatography-negative ion chemical ionization mass spectrometry and has a sensitivity limit of 500 pg ml^{-1} " should read "... a sensitivity limit of 500 cg ml^{-1} ".

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ARTICLES

Geology and stratigraphy of Neogene deposits, Middle Awash Valley, Ethiopia

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The Middle Awash Valley, Afar, Ethiopia, contains a sedimentary sequence that is >1 km thick, spans much of the Neogene, and contains vertebrate fossils throughout. Newly defined formations described here are older and younger than the hominid-bearing Hadar Formation. The Awash deposystem reflects deposition in an evolving triple junction.

THE Awash River Valley in the Afar Depression (Fig. 1) is unique for containing a 'triple junction river'¹, and, as reported here, a sedimentary record >1 km thick (Fig. 2) that spans much of the Neogene history of the region, including the final breakup of Afro-Arabia. Fossil remains occur throughout the sequence, including those of early hominids. Either hominid fossils or artefacts occur throughout >0.5 km of strata, from the lower Pliocene levels at Hadar² to Pleistocene strata at Bodo^{3,4} and Andalee⁵ (Fig. 2).

This report is based on six seasons of fieldwork with intervening studies by the Rift Valley Research Mission in Ethiopia^{1,3-9} (RVRME), under the direction of one of us (J.K.), from early 1975 to mid-1978. Geological investigations are reported below. The accompanying article¹⁰ will report palaeontological and archaeological findings as well as the age of the deposits.

The area studied lies between volcanic plateaus within the Middle Awash Valley (delimited at Awash Station and Tendaho) between Gewani village and Hadar (Fig. 1). Previous geological work in the area is limited to preliminary geological mapping along the SE margin of the area^{11,12}, observations near Gewani¹³, studies at Hadar^{1,14,15} and photogeology^{13,14,16}.

The area studied by the RVRME extends from Gewani to 10°57'N (Fig. 3). The area was geologically mapped from 1:60,000 air photos. This includes the area north to Hadar (from studies by J.K. and D.P. in 1972-73 and traverses by J.K. and A.M. in 1975-76). Extensive base map preparation (aided by H. Mosca and W. Singleton) preceded field surveys. Some 120 geological profiles taken in the map area are referred to in Figs 4 and 5 and in the text by profile numbers (P.1,2,3, and so on); elevations are estimated from published sources¹⁷. Geographical names are from Afar inhabitants (those in quotation marks are provisional).

The map area (Fig. 3a, b) lies within a northeastward-drained trough bounded to the west by the Ethiopian Escarpment made up largely of the Miocene 'Fursa basalts'¹⁸, and to the east by a basalt plateau comprised of the Pliocene 'Upper' Afar Stratoid Series^{19,20} (UASS). The south-central area (Fig. 3a) is capped by basalts of the 'Lower' Afar Stratoid Series (LASS). This central area is complexly faulted and referred to as the Central Awash Complex (CAC) (Fig. 3a); it contains the 'Hatowie Graben', bounded by the 'West Hatowie Horst' (WHH) and the 'East Hatowie Horst' (EHH).

The map area forms a northward-dipping structure (due to greater subsidence towards the triple junction) with margins blockfaulted towards the central part of the basin and to the north: older strata crop out to the south along basin margins and the CAC (Figs 3, 5). Dips are greatest at basin and graben

margins (or zones of maximum uplift), generally in older strata, and are least pronounced in Pleistocene deposits. Fault trends generally conform to the NW-SE Ethiopian (East African) Rift trend (Fig. 1). Closer inspection reveals the NW trend becomes generally more westerly to the north, and the NE trend more easterly to the south (Figs 3a, b), a reflection of the rotational movements of the Arabian and East African plates (see refs 21, 22).

Typical badland topography in the area forms exposures up to 20-40 m along basin margins to the south, up to 15-20 m on the EHH, and 90-100 m to the north, where strata are deeply incised by the Awash River.

Stratigraphy (Awash Group)

Stratigraphical studies to date are contained in reports to the Ethiopian Government, in regional, site or faunal descriptions^{1,4-9}, or will shortly appear in monographic form²³. Below we summarize the studies and provide the first regional stratigraphical framework for the Middle Awash, as well as briefly describe individual stratigraphical units. [For reference, the Mio-Pliocene boundary is established at 5 Myr (ref. 24), the Plio-Pleistocene boundary at about 1.6 Myr (ref. 25) and the middle Pleistocene boundaries at about 700,000 yr and 125,000 yr (ref. 26).]

The Awash Group is defined here as all late Cenozoic continental sedimentary strata within the catchment of the Awash River. As described below, its known thickness is >1 km thick, and includes the late Miocene Chorora Formation²⁷ on the SW Hararghe Escarpment (Fig. 1) and the Pliocene Hadar Formation², and the presently-described Adu-Asa, Sagantole, Matabaietu and Wehaietu Formations (Fig. 2).

Adu-Asa Formation: Estimated to be late Miocene to early Pliocene^{8,10}, with a minimum thickness of 145 m; it is best exposed in the SW map area (Fig. 3a), between 725 and 825 m a.s.l. (above sea level), and in the CAC from ~640-660 m a.s.l. Adu refers to Adu Dora (white place stream); Asa refers to Asa Koma (red hill). Units comprising the formation (from bottom to top) are the Adu, Asa, and Kuseralee Members (Fig. 2).

Adu Member: At least 40 m of strata either in fault contact or unconformable with the 'Fursa basalts'¹⁸ (P.2,3). The lower unit, especially prominent at upper Ananu and Adu Dora N. (P.3,4), consists of up to 30 m of variegated, predominantly pinkish clays and silts with some variable, cross-bedded sands (up to 2.5 m) and yellow-green clays. The upper unit consists of 12 m or more of massive diatomites (up to 2-3 m), white,

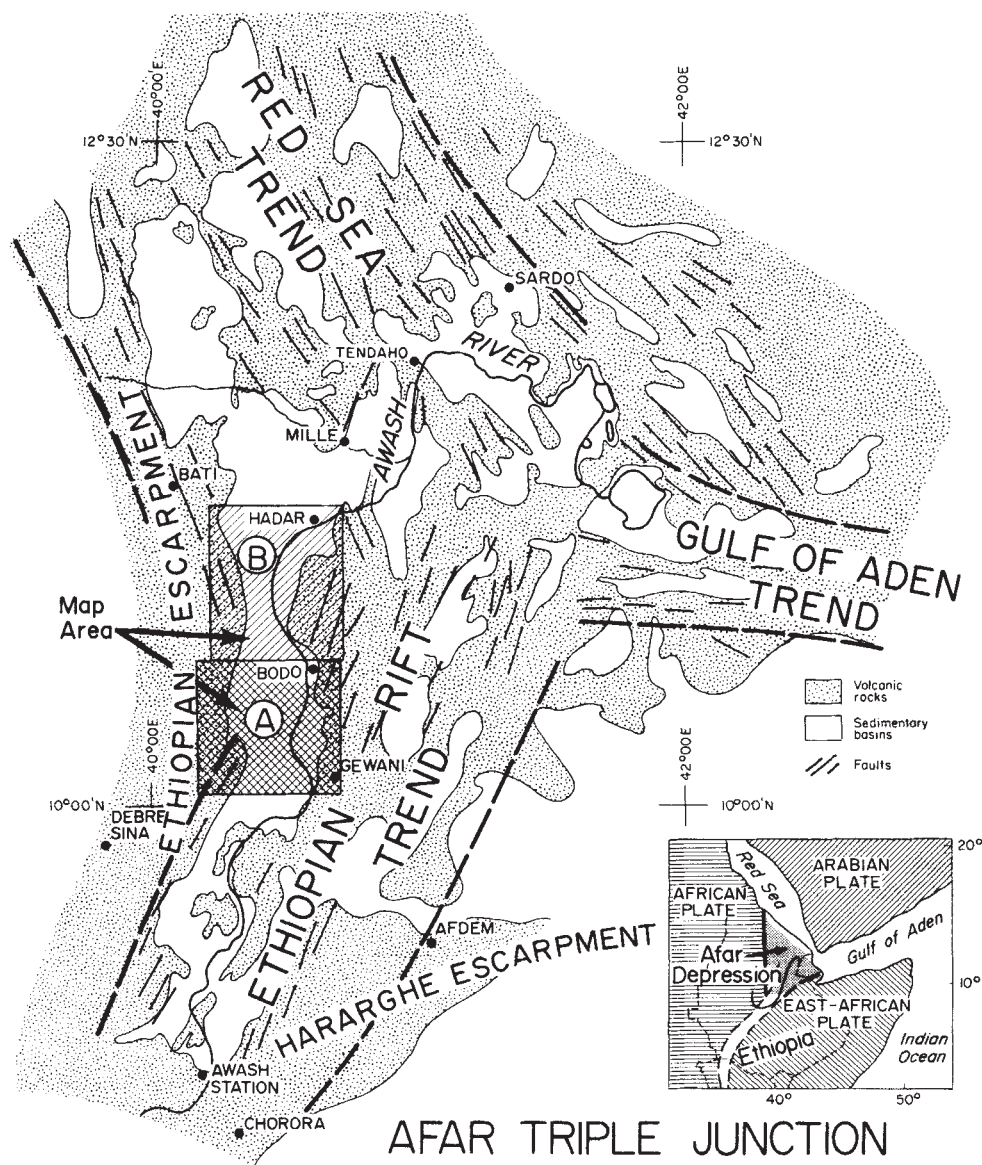


Fig. 1 Study area location map.

indurated clay with some fish bones, reddish-brown clay, and some grey, laminated tuffs.

Asa Member: At least 75 m of strata overlying the Adu Member. The base of the lower unit is marked by either an erosional surface containing distorted bedding and gravel lenses (P.4,6) and/or prominent tuffs. At Asa Hill (P.5), this unit consists of up to 33 m of waterlain well-stratified tuffs (1–3.5 m thick), reddish-brown clays, some coarser layers and minor diatomite lenses. At Adu Dora N. (P.7), the upper unit consists of 30–40 m of predominantly reddish-brown clay, with discontinuous sand (up to 1.2 m), silt and thin tuff layers. Elsewhere, this unit may be overlain by (LASS?) basalts or unconformable alluvium. Fossiliferous strata that may be equivalent to this member exist at Saitune Dora (Fig. 3a).

Kuseralee Member: Up to 30 m of strata beneath the LASS basalts, especially at Kuseralee and elsewhere along the margins of the Hatowie Graben (Fig. 3a). The stratigraphical position of this unit to the Asa Member is based on its downfaulted lower altitudinal, central position in the basin (P.9,13) and by its more derived fauna. Between Kuseralee and Koba-ah Streams (P.12,13) are up to 30 m of mauve to pinkish tan, sometimes grey, clays with some silts and channel sands and sandstones (up to 3.5 m). Clays form a baked contact zone subjacent to the basalts. Equivalent strata may occur in the

eastern Awash between Asaberi and As Streams (Fig. 3a), beneath thick (LASS?) basalts (P.61).

Sagantole Formation: Estimated to be early Pliocene^{8,10}, with a thickness of 210–226 m (Fig. 2) overlying the LASS basalts. Best exposed in the eastern CAC between 757 and 660 m a.s.l. (Fig. 3a, 5). We divide this formation into the Haradaso and Aramis Members and two informal units, the Bearyada and Kalaloo beds (Fig. 2).

Haradaso Member: From 71 to 76 m. The lowest strata preserved on the LASS basalts may be in down-faulted structures on the EHH at Haradaso (P.14) and elsewhere. Here are 10–15 m of predominantly reddish-brown and grey clays, with some silts, sands and vertebrate remains¹⁰. The main unit is in depositional (locally conformable?) or fault contact with the eastern CAC basalt margin and extends 1–2 km eastward between Aramis and Sagantole S. Streams (P.16,17). Here up to 61 m of strata consist of four sand and clay units. Sands are 2.5–6.5 m thick, dominantly fine- to medium-grained, may be laminated or contain coarser lenses, but are commonly homogeneous and interdigitated with tan, reddish-brown or grey clays, silt and shale (with fish remains). At mid-sequence, a lignitic or carbonaceous shale (5–10 m) (P.16,17) exists within a grey (partly tuffaceous?) clay. Upper sands are brown, contain

coarser layers, and are associated with brown clays and silts. Equivalent strata in the eastern Awash may exist between Sibabi and Asaberi (P.62,64) overlying (LASS?) basalts, and at lower Wee-ee (P.65).

Aramis Member: At least 50 m of strata unconformable or in fault contact (?) with the Haradaso Member (P.18,20). This unit is distinguished by the first major appearance of tuff, which occurs throughout this (and overlying) units. At Sagantole S. are at least 50 m of: (1) tan silts (12.5 m) with siltstones and tuffs (up to 1.75 m); (2) tan, orangish silt (24 m) with sand and tuff layers—upward becomes increasingly sandy (up to 88 m) with gravel lenses; (3) light tan silt (11 m) with sandstone lenses. Equivalent beds in the eastern Awash may exist at lower Wee-ee (P.65).

Beeryada beds: Thickness ~75–85 m (exact values are unknown owing to faulting). Between Aramis S. and Sagantole N. (P.20,23), these beds conformably overlie the Aramis Member and consist of: (1) well-bedded yellow tuffs (4 m+) (P.20); (2) pinkish to tan silts with grey, well-stratified, laterally continuous tuffs (2 cm–1.5 m), and some sand, sandstone and clay (30–40 m); (3) at Beeryada, tan silt and sand below and coarsening upward sand and conglomerate above (11.5 m); (4) repeat of (2) above (5.3 m); (5) brown clay, silt and some sands, tuff and brown mudstone (15–18 m). These beds are capped by laterally continuous, gastropod limestone layers (P.23). Strata possibly equivalent to the upper part of this unit in the eastern Awash lie below a widespread gastropod limestone between Kalaloo and Bodo Streams nearest the basalt (P.24,25), and south (Fig. 3a).

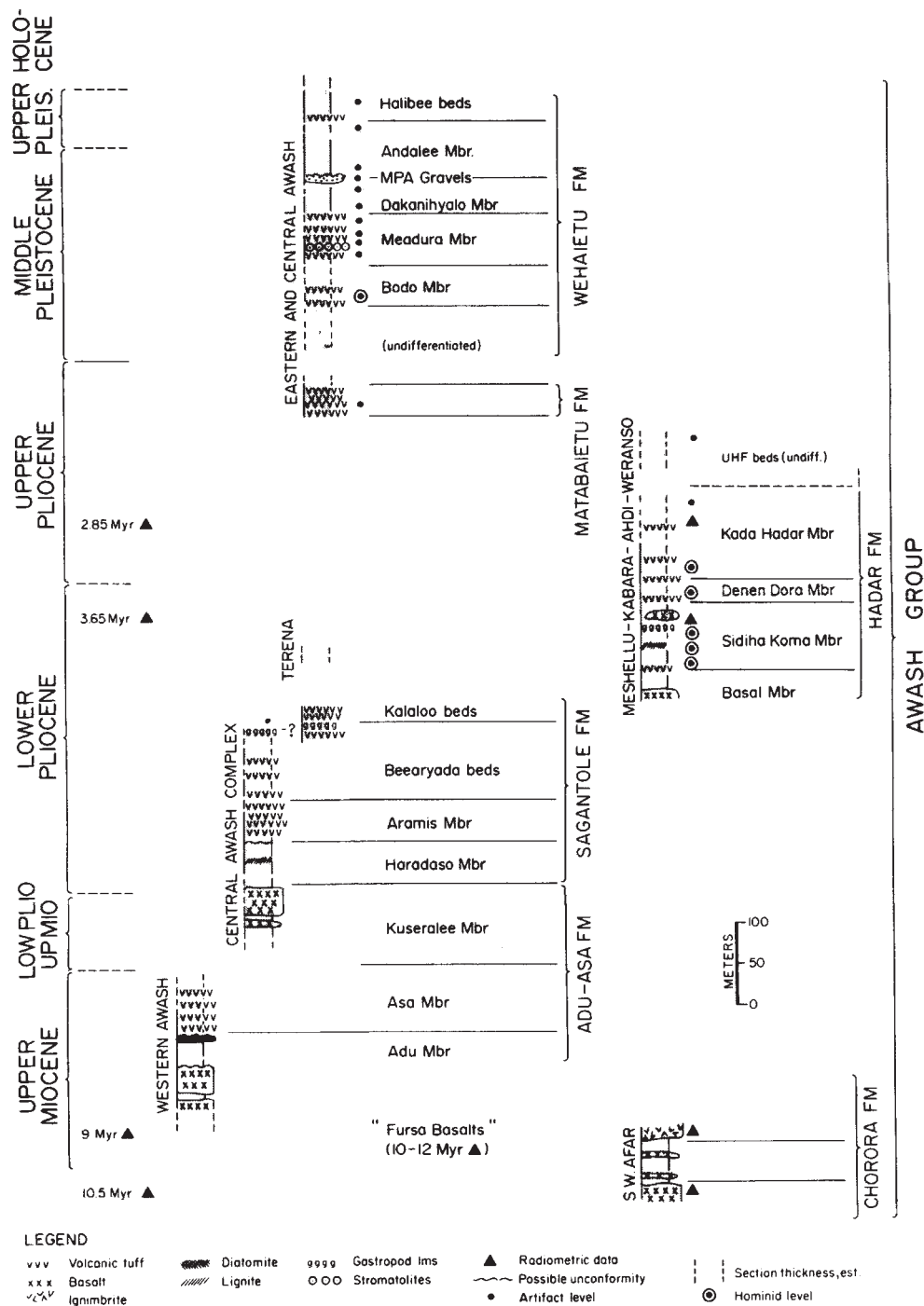


Fig. 2 Stratigraphy of the Awash Group.

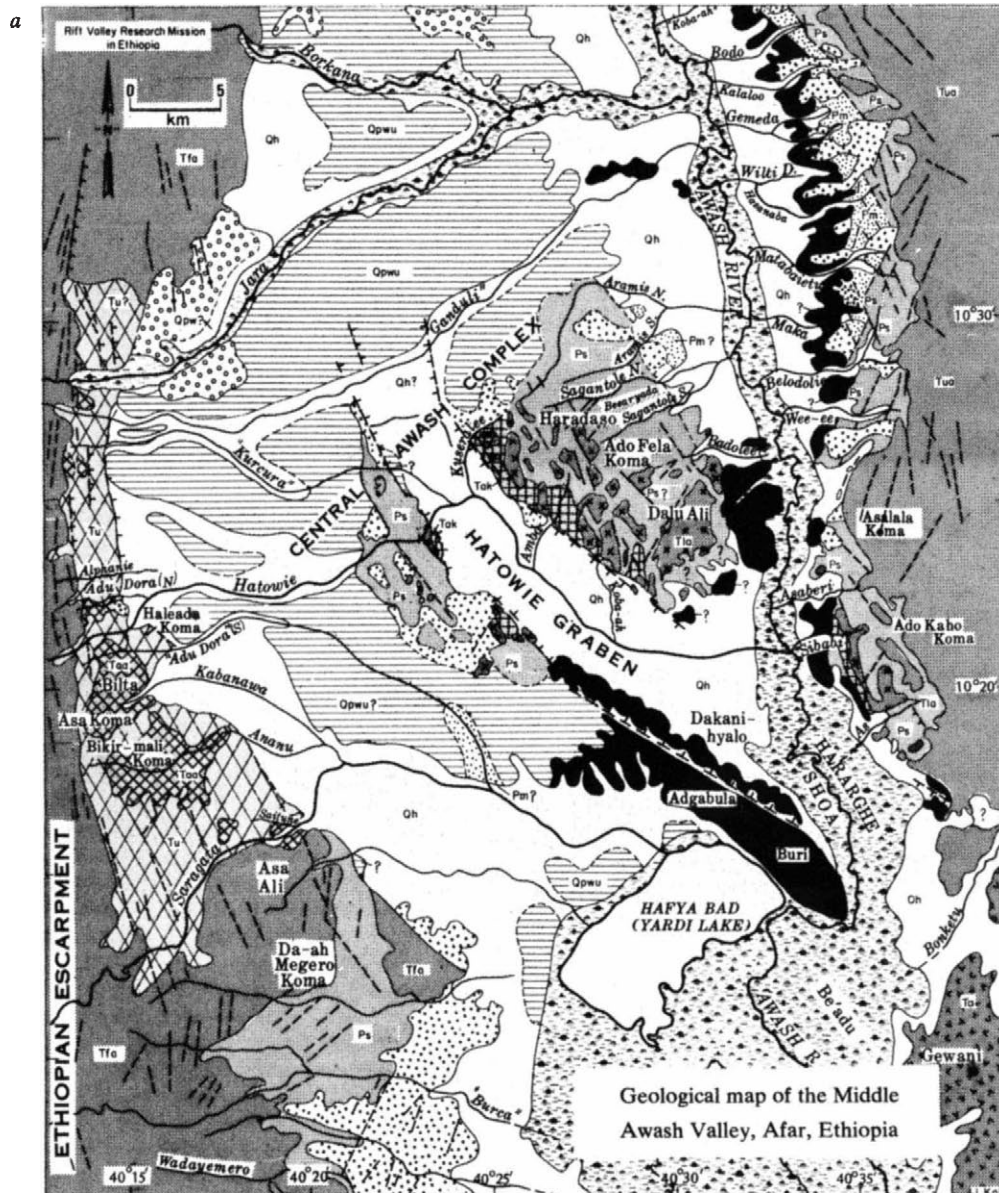
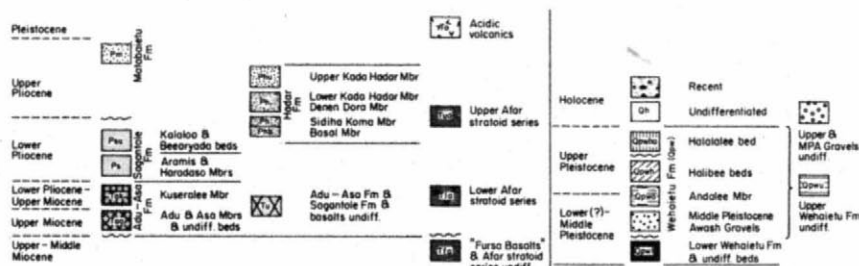


Fig. 3 a, Geological map of the Central Middle Awash Valley (southern sector). b, Geological map of the Central Middle Awash Valley (northern sector).



Kalaloo beds: A minimum of 14–15 m of strata. Overlying the gastropod limestone (P.24–26)/between Kalaloo and Bodo Streams, the base is marked by a widely traceable coarse or lapilli tuff (10–35 cm), followed by a variable sequence of tan silts with siltstone, sand, and microconglomerate lenses, with grey and brown clay upwards. Several tan, silty tuff layers (0.5 m to 3.5 m in a channel at mid-sequence) are present. Some disconformable strata may exist in the upper portion.

Hadar Formation: Predominantly middle Pliocene^{10,28,29}, and up to 270 m thick, this unit crops out well north of the Sagantole Formation (Fig. 3b), and, because of regional subsidence, at lower elevations (P.40,48). If physical correlation is possible between the Hadar and Sagantole Formations, it would prob-

ably require tracing the Kada Damoumou basalt³⁰ (P.46) to basalts further south that intercalate the Sagantole Formation. The Kada Damoumou basalt seems to correlate with basalt to the SE; correlation further south is complicated by erosion of overlying beds, and intervening faults and younger beds. Basalt in a stream-bed ~5 km SW of the Ounda Meshellu–Awash confluence appears to lie at the base of the Hadar Formation (P.40) (Fig. 2).

Upper Hadar Formation (UHF) beds, undifferentiated: At least 50 m thick, these beds crop out north of Hadar in the drainages of Ledi, Geraru and Weranso Streams and the Mile River^{14,15}. These beds, first surveyed in 1971–74 by three of us (J.K., D.P., E.O.), and in part by Taieb^{14,15}, occur downp-

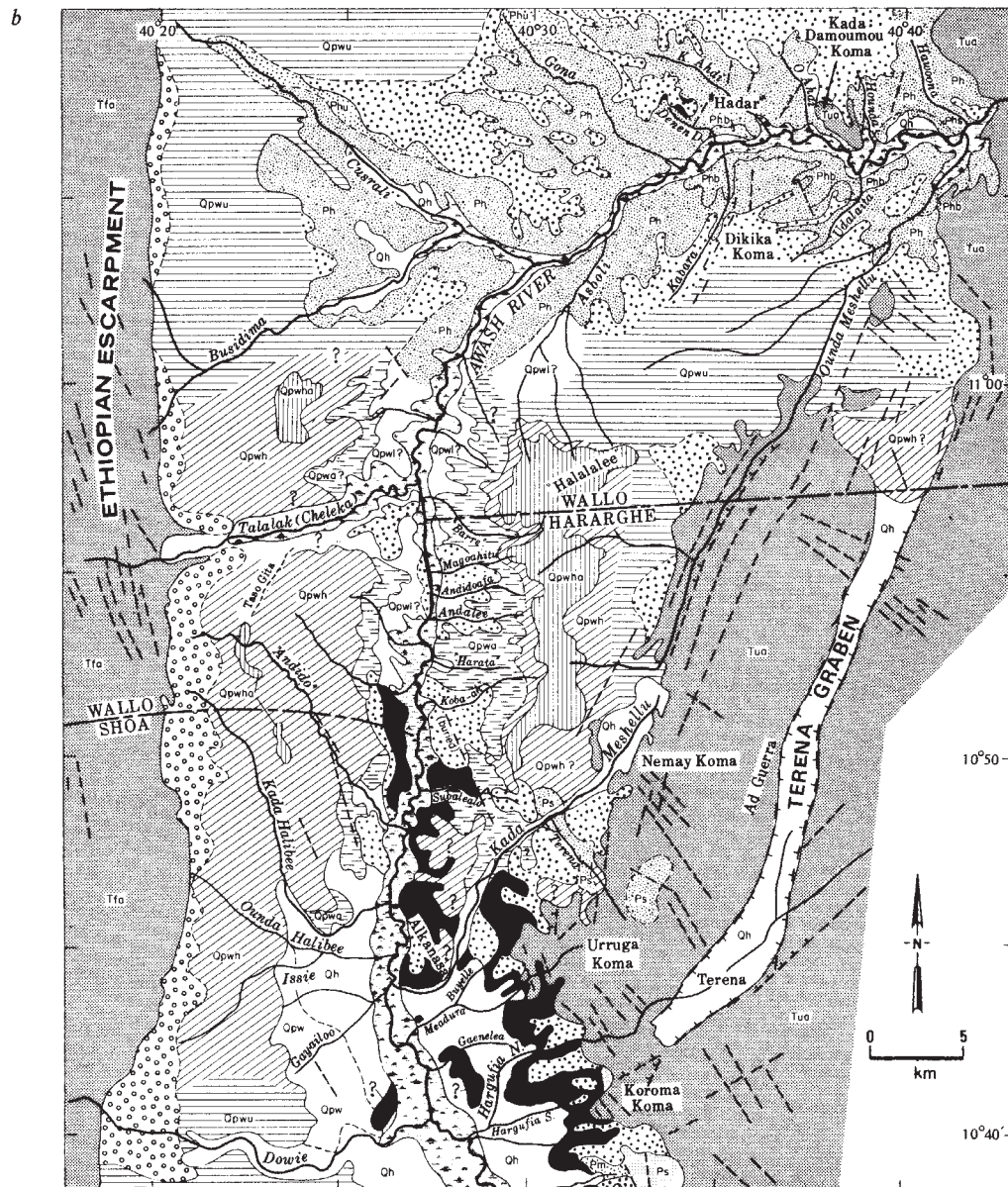


Fig. 3 (cont.)

or are downfaulted in relation to the Hadar Formation (P.48). They are broadly similar to the described strata in the upper Hadar Formation, but contain fauna associated with younger units¹⁰.

Matabaietu Formation: Late Pliocene¹⁰, at least 52–57 m thick (Fig. 2), and best exposed in the eastern Awash (Fig. 3a). In fault contact or unconformable with the Sagantole Formation (P.70,73). At Matabaietu, the lower unit consists of 34–39 m of up to three variable, discontinuous, cyclic units of clay, sand and tuff deposition. At the base are predominantly reddish-brown clays (23–28 m thick), with some mottled darker clay. Sands (up to 2 m) are irregularly bedded, calcareous and contain gravel lenses, cross-beds and plant casts. Tuffs are up to 2.5 m, sandy or silty, reworked, well-weathered and contain gravels, plant casts and limestone concretions. The upper tuff, from which an Oldowan chopper was recovered¹⁰, is referred to as the 'chopper tuff'. The upper unit (14 m) outcrops west and up dip from the chopper tuff, which it appears directly to overlie, although a fault contact is possible. Strata consist of: tan and grey clays (12 m) with small concretions and plant casts; argillaceous tuffs with plant casts; and some microconglomerate lenses. The unit is capped by up to 4 m of (unconformable?) pebble conglomerate and minor brownish-grey clay. Similar strata exist in the uppermost levels of Hadar and in the UHF

beds. Based on faunal considerations, the upper UHF beds may be of similar age¹⁰.

Bikirmali beds: Up to 30–40 m or more of poorly-sorted tuffaceous alluvium truncate the Asa Member at Bikirmali Hill (P.6) (Fig. 2a) and elsewhere. The stratigraphical position of these beds is uncertain; however, a late Pliocene age is inferred by fragmentary vertebrate remains (*Hexaprotodon*, progressive *Elephas recki*)¹⁰. Basic basalts (7–8 m) cap the sequence.

Lower Pleistocene (?) Awash conglomerate: It may be that only minor deposits document the period between the Matabaietu Formation and the Bodo Member (Wehaietu Formation). Their faunas clearly show the age difference between these two units¹⁰. Missing strata may be explained by faulting, and in part by erosional episode at the end of Matabaietu deposition, which may possibly be recorded by uppermost conglomerates at Matabaietu and Bodo; north of Hadar, conglomerates cap the UHF beds at various localities, perhaps the most prominent being at As Hill, 15 km NW of Hadar. Here massive (4–6 m thick), resistant conglomerates, some 35–45 m above the surrounding plain, truncate the UHF beds,

Lower (?) to Middle Pleistocene beds, undifferentiated: At Wilti Dora, a 3–4 m waterlain tuff overlies the Matabaietu Formation unconformably and is downthrown to the west by

a normal fault. This tuff contains some fossils, including: *Kolpochoerus* sp., hypsodont *Hippopotamus* sp. and *E. cf. recki*, and the first appearance of *Equus* in the Awash Group. West of and apparently overlying the 'Equus tuff' are >14 m of largely unfossiliferous brown clays, with some conglomerate layers that extend southward and may be the same as sediments (estimated at 20–30 m thick) west of the conglomerates capping the Matabaietu Formation at Matabaietu. At Bodo, as at Wilti Dora, a thick tuff (Equus tuff?) appears to overlie the Matabaietu Formation unconformably and is offset by faults. Fossils from this tuff are *Equus* and a large *Theropithecus* (similar to a robust form from the Bodo Member) (C. Jolly, personal communication).

Wehaietu Formation: Early (?) to late Pleistocene¹⁰, with an estimated thickness of 200–250 m. Exposed throughout the

map area (Fig. 3a, b), between 500–650 m a.s.l. (Fig. 5). Wehaietu is a name in the Afar language used interchangeably for the Awash River. This formation is divided into (from bottom to top): the Bodo, Meadura, and Dakanihyalo Members, the MPA Gravels, the Andalee Member, and the informal Halibee beds (Fig. 2). The MPA gravels divide the formation into two readily distinguishable larger units, the lower and upper Wehaietu Formation.

Bodo Member: At least 16–19 m of strata between the Bodo and 'Koba-ah' Streams (Fig. 3a, b). The base of the member, as presently defined, consists of the previously-described Upper Bodo Sand Unit⁴ (UBSU) (P.28) that unconformably overlies thick, reddish-brown clays (Matabaietu Formation). Acheulian artefacts and fossils, including an archaic *Homo* cranium³, are abundant throughout the lower half of the unit¹⁰. North of Bodo, the USBU is overlain by brown clays containing (barring any intervening faults) tuff lenses that wedge out to the west.

Meadura Member: A minimum of 45–50 m of strata between Hargufia and Meadura Streams (Fig. 3b). These strata have not been correlated directly with the Bodo Member because of intervening alluvium; however, because of lithological differences, the northward regional dip (almost certainly placing the Meadura beds higher stratigraphically) and faunal distinctions¹⁰, these strata are treated as a separate unit. At Meadura, the lowermost strata may be those to the far east, altitudinally higher than the main (downfaulted?) outcrop areas to the west, in marginal grabens, and in adjacent areas to the south. To the west, a minimum of 35 m strata lie unconformably against basalts, or are downfaulted. Lithologies exhibit considerable lateral facies variation and are dominantly brown (sometimes mottled grey) and tan clays, tuffaceous silts and tuffs (0.6–1 m); fine- to coarse-grained sands (1–4.6 m) with gravel lenses; and poorly-sorted sandstone conglomerates (0.7–1.4 m). Tuffaceous deposits are concentrated in the upper sequence, as is abundant calcium carbonate material. The latter includes spheroidal stromatolite boulders (up to 1.5 m in diameter) with laminations and micro-structure similar to bacterial stromatolites (R. L. Folk, personal communication); these can be traced southwards to Hargufia⁴. Strata are broadly similar at Hargufia but may extend lower stratigraphically (P.29,30).

Middle Pleistocene beds, undifferentiated: Estimated 25–35 m of strata. At Buyelle (P.31), the Meadura Member is overlain by at least 15 m of predominantly orangish-brown, fine-grained sediments that abut basalts (Fig. 3b). Between 'Alkanasa' (P.33) and 'Subalealo' (P.35) are a minimum of 10–15 m predominantly sandy sediments with tuff layers. Thick, well-bedded tuffs, tan silts and clays occur at the base of the Subalealo sequence; to the north, these beds appear truncated by the MPA Gravels; immediately south, they may be overlain unconformably by the upper Wehaietu Formation (?). Fauna from Subalealo is consistent with that in the Meadura Member. In the Hadar area, terraces wedged between the Hadar Formation and the MPA Gravels, bearing Acheulian tools^{31,32} (P.46a), may be assigned to the lower Wehaietu Formation.

Dakanihyalo Member: At least 15–20 m of strata that occupy the SE portion of the WHH (Fig. 3a). The higher stratigraphical position of this unit relative to the Meadura Member is inferred by its central and generally lower altitudinal position (P.56,57) in the Awash basin, and its associated fauna¹⁰. At Dakanihyalo and to the west, sediments are commonly buff and reddish-brown discontinuous beds dominated by medium to very coarse, cross-bedded sandstones (locally up to 5 m thick), and pebble conglomerates, with silt and clay interbeds.

Middle Pleistocene Awash Gravels: 1–4 m thick, these gravels are ubiquitous in the Middle Awash³¹ (Figs 2, 3a, b) truncating the lower Wehaietu Formation or older units. They are named the MPA Gravels to distinguish them from late Pleistocene and Holocene gravels³³ as well as from older gravels. Their relationship to surrounding units is best seen between Ounda Meshellu and 'Barre' Streams (Fig. 3b).

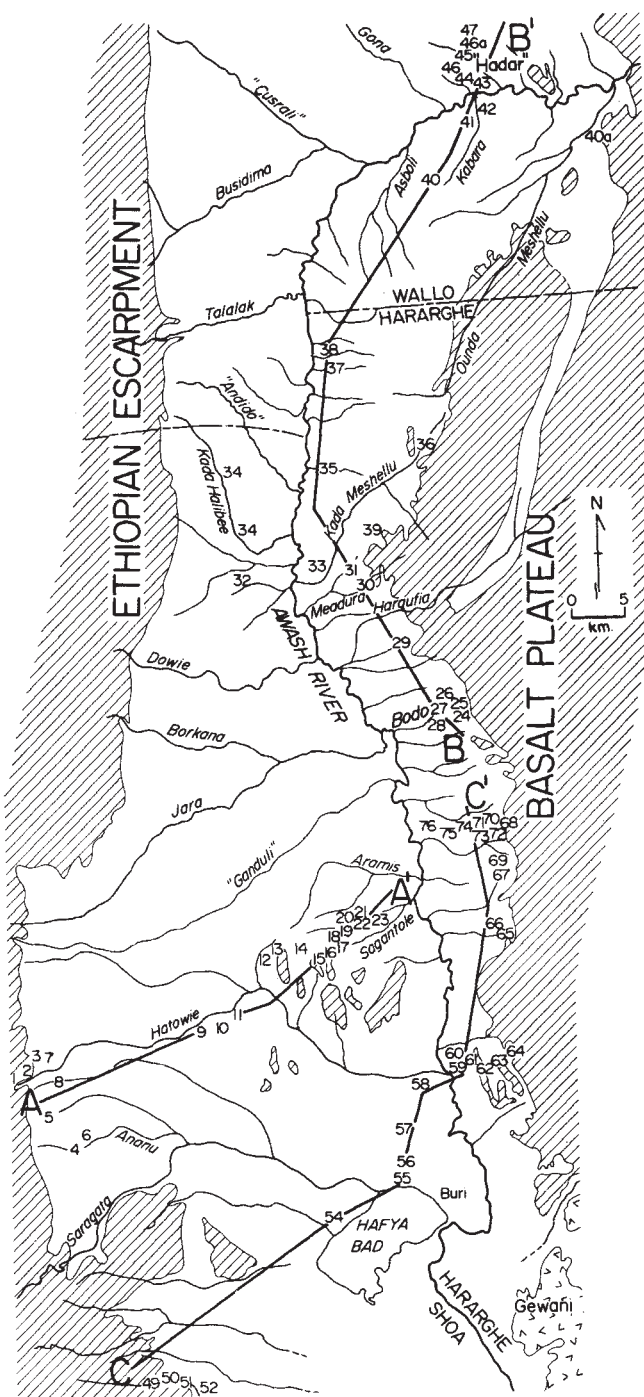


Fig. 4 Geological profile index map.

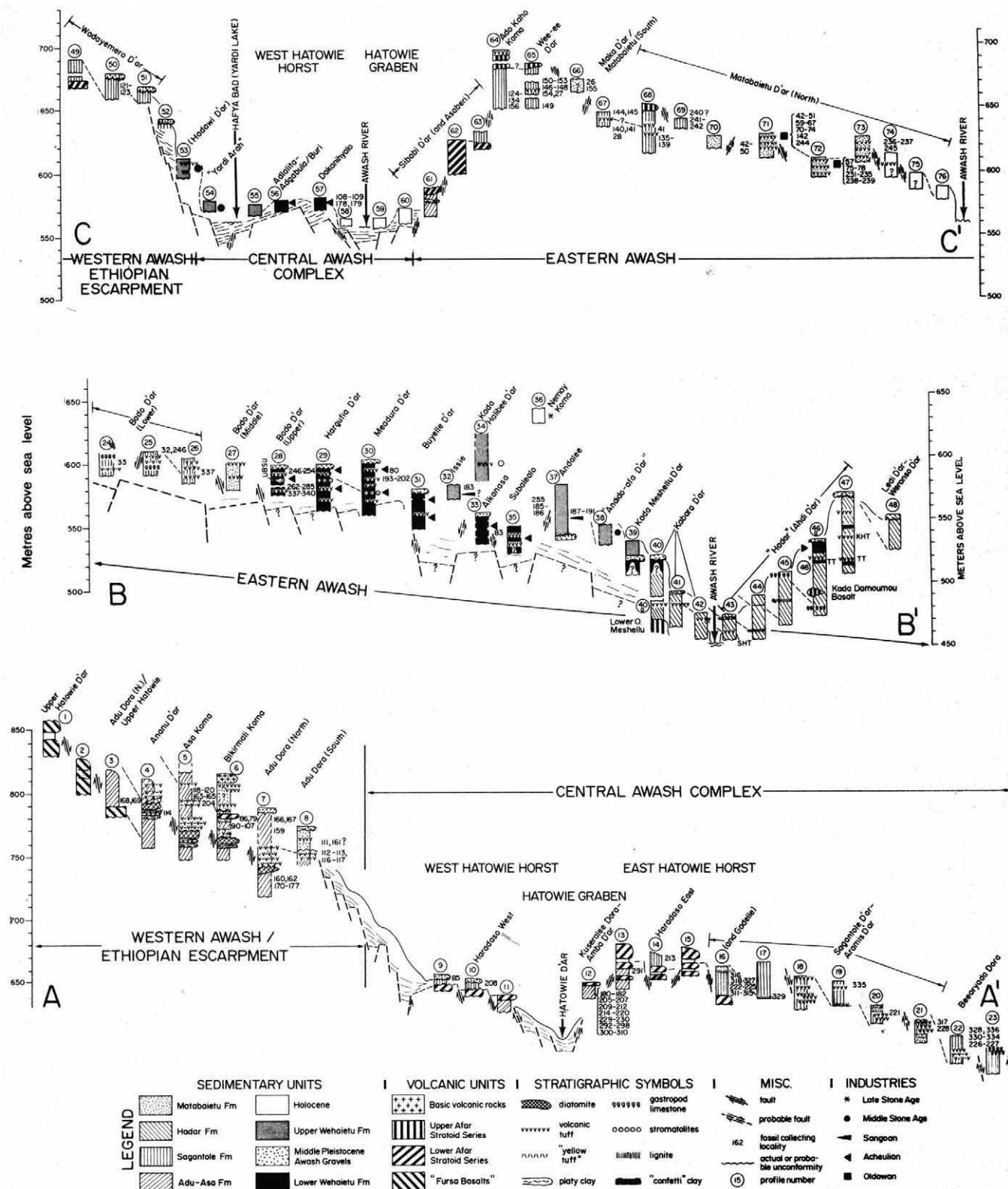


Fig. 5 Composite geological profiles of the Middle Awash Valley.

Andalee Member: Estimated to be a minimum of 55–60 m thick. In the northern map area (Fig. 3b), these strata appear to onlap progressively the northward-dipping MPA Gravels, forming an unconformable wedge that thickens to the north (and in the central part of the basin). At Andalee, a preliminary assessment of the local geology has been made⁵. The lower sequence rests on the MPA Gravels (P.37) and consists of 6–8 m of sand, silt and clay interbeds with calcified concretions, micro-

conglomerate lenses, and a rich concentration of very small- to medium-sized vertebrates, associated with a Sangoan-like tool industry^{5,10}. The upper sequence is >34 m thick and consists of massive brown clay and silt with cross-bedded sands and gravel lenses.

At Issie, in beds that appear to lie just below the basal tuff (?) of the Halibee beds, is another locality rich in very small- to medium-sized vertebrates associated with a diverse tool

industry^{5,10}. These are contained within 2–3 m of tan loosely-consolidated silts and clays with minor sands.

Halibee beds: Estimated to be up to 35–45 m of strata conformable with the Andalee Member (P.34), as best observed between Kada Halibee Stream and the Talalak River (Fig. 3b). These beds are undifferentiated and informally defined. Generally, at the base seems to be a widely traceable tuff layer, overlain by predominantly light-coloured, fine-grained, loosely-consolidated sediments containing some tuffs and darker clay, sand and gravel layers.

Halalalee bed: Mantling the Halalalee plain (Fig. 3b), at the top of the Halibee beds, is 1–2 m of unconformable, dark-brown and grey clayey layer that may be a palaeosol. Only remnant patches of this bed occur in the western Awash, overlaying the upper Halibee beds.

Holocene beds: Deposits that postdate the Halibee beds, and contain late Stone Age artefacts¹⁰ and vertebrate remains, occur throughout the Middle Awash (Fig. 3a, b) at progressively lower altitudes, between ~645 and 490 m a.s.l. At Nemay Koma, in the uppermost catchment of Kada Meshellu Stream (Fig. 3b) (P.36), between ~630 and 645 m a.s.l., are at least several metres of predominantly tan fine-grained sediments unconformably overlying Pliocene basalt and strata. Along the SE margin of the map area near Gewani (Fig. 3a) are 4–8 m of deposits that represent a lake terrace at least 575 m a.s.l. These deposits are diatomaceous and rich in calcium carbonate and stromatolitic material¹³. At Mero Buri, south of Hafya Lake some 14–16 km, are 3–4 m of light-brown silts and sands occurring several metres above the modern Awash floodplain.

Palaeoenvironments

The base of the late Miocene Adu-Asa Formation is marked by fluvio-lacustrine deposition in the lower Adu Member. Massive diatomite formation in the upper Adu Member indicates distinct lake development. The end of the Adu period is marked by erosion (localized?) and an abrupt onset of massive tuff deposition in the Asa Member, probably associated with widespread volcanic (and hydrothermal) activity, regional subsidence and an eastward migration of lacustrine deposition. This period may have coincided with or slightly preceded one of widespread tectonism and renewed spreading in the southern Red Sea in the late Miocene³⁵.

The thickness and stratification of the tuffs in the Asa Member combined with minimal diatomite formation and fluvial layers, indicate that the Adu-Asa lake environment probably entered a period of instability, decreasing depth and increasing fluvial influences. The upper Asa Member suggests a return to alluvial conditions and a tapering off of volcanic activity. The Kuseralee Member indicates predominantly fluvial deposition; this was interrupted, and the Adu-Asa Formation sealed, by massive extrusion of LASS basalt lavas during the latest Miocene to earliest Pliocene¹⁰.

Strata comprising the bulk of the Haradaso Member suggest a dominantly progradational depositional pattern (lake margin, marsh, stream) that may reflect adjustments to regional subsidence. The Aramis Member and Bearyada beds appear dominated by shallow and marginal lacustrine environments, with episodes of fluvial and fluvio-deltaic deposition; the Kalaloo beds indicate predominantly lake margin and alluvial deposition. Tuffs throughout these units indicate continuous volcanic activity, as do intercalated UASS basalts.

The Hadar Formation generally reflects a northeastwards migration of the Awash deposystem during the Pliocene, indicated by a pattern of basin expansion at Hadar during the middle Pliocene, and progradation in the late Pliocene (cf. ref. 28). This accompanied the well-documented period of extensive graben development and subsidence in the central Afar during the Pliocene (see refs 21, 22).

The predominantly fluvial deposits in the upper Hadar Formation also appear dominant in the UHF beds and the Matabaietu Formation (and the Bikirmali beds) and point to a prolonged period of alluviation and some downcutting in the late Pliocene, accompanying a change in base level, tectono-volcanic activity, an eastward migration of lacustrine deposition (especially evident at Geraru¹⁵) and generally drier conditions. This period may have been followed by a major episode of clastic deposition and erosion in the early Pleistocene.

It is clear that by the time of deposition of the lower Wehailu Formation in the early (?) to middle Pleistocene, the Middle Awash had undergone major structural and geomorphic changes with deposition concentrated at distinctly lower elevations and further north. Whereas the fluvial deposits at Bodo⁴ and the Acheulian tool-bearing terraces at Hadar indicate alluviation and downcutting, the calcium carbonate-rich deposits and stromatolites stretching from Hargufia to Meadura indicate a period of widespread transgressive lacustrine deposition. The texture of the stromatolites suggests bacterial growth enhanced by geothermal waters (see, for example, ref. 36); their spheroidal shape suggests a lacustrine setting similar to that described for stromatolites near Lake Turkana³⁷. Strata overlying the Buyelle-Alkanasa beds, including the Dakanihyalo Member, suggest predominantly fluvial deposition. Associated tuffs throughout the lower Wehailu Formation indicate continued volcanic activity, probably from silicic centres near Gewani^{19,20} and elsewhere; likewise, faults and tilted strata indicate tectonic activity.

The MPA Gravels indicate major peneplanation and extensive downcutting during an apparently prolonged and major dry period, probably during the late-middle Pleistocene³⁸. At this time, strata were heavily eroded, and an extensive, northward-sloping pediment was created, such that the upper Wehailu Formation overlapped the MPA Gravels from north to south. The lower Andalee Member (and its associated fauna) suggests deposition initially occurred in a sub-arid environment much like the modern Awash Valley⁵. Following deposition of the Halibee beds and continued tectono-volcanic activity, the Awash was again subjected to regional downcutting. This began apparently at the end of the Pleistocene and continued during successive dry periods during the Holocene (compare with refs 33, 34, 38). Intervening wetter periods seem to be indicated by (lacustrine?) deposits at Nemay Koma and near Gewani. Those near Gewani are equivalent in age to transgressive lake deposits described in the central Afar^{13,33}. An early Holocene lake in the Gewani area would extend possibly over much of the depressed area extending from Sibabi to the basalt plateau 44 km to the south (Fig. 3a).

The palaeogeography of the Middle Awash indicates a deposystem that has continuously migrated towards lower and more northeasterly positions from the late Miocene to the present. Basin development was in concert with subsidence and rotation of host structures controlled by crustal attenuation and plate movements in the triple junction. This interaction of deposition with tectonics is reflected in the movement of lake basins to the NE, as well as by major changes in base level that amplified the effects of erosion and downcutting during dry periods.

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Fossil mammals and artefacts from the Middle Awash Valley, Ethiopia

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A review of fossil mammalian faunas from the Middle Awash indicates they span most of the later Neogene and document evolutionary change in several mammalian groups, especially Primates, Proboscidea and Artiodactyla. Oldowan artefacts first appear in the late Pliocene, while Acheulian and later industries and apparent occupation sites occur in Pleistocene beds.

INVESTIGATION of the Middle Awash deposits by the Rift Valley Research Mission in Ethiopia^{1,2} (RVRME) resulted in the documentation of 333 fossil localities and hundreds of archaeological occurrences. Fossil and artefact localities were plotted on air photographs (1 : 60,000) and/or site maps (1 : 6,000) and in stratigraphical sections that permitted them to be precisely located geographically and inserted in the regional geological sequence. Detailed locality maps and reports were submitted to the Ethiopian Government^{3,4}.

Approximately 2,500 fossils were collected and studied in the Ethiopian National Museum and the Paleobiology Research Laboratory at Addis Ababa University. Only limited samples of artefacts were recovered. Fossils and artefacts are stored in the Ethiopian National Museum. Many additional fossil specimens, especially skulls of large ungulates that were too bulky to be collected with the resources at hand, were left *in situ* (and often photographed) for future collection. When added to the

previously described data from the Hadar Formation, these collections provide a biostratigraphical sequence that documents long stretches of the evolutionary history of the Middle Awash fauna since the late Miocene.

Fauna and biostratigraphy

Mammalian taxa identified in the collections are systematically listed in Table 1 and are designated according to stratigraphical unit. Table 2 provides the distribution of major taxa according to the order of their first appearance in the regional sequence, inclusive of taxa from the Chorora⁵ and Hadar Formations^{6,7}. Most work has been carried out on the primates, Proboscidea and Artiodactyla. Previous accounts of the various vertebrate taxa are contained in reports to the Ethiopian Government^{3,4}, regional summaries^{1,8}, site reports^{9,10}, and studies of individual taxa^{11–13}. More detailed treatment will be reported elsewhere¹⁴.

Table 1 Systematic faunal list, Awash Group (newly described units only)

Mammalia		Perissodactyla
Chiroptera indet. (As)		Equidae
Primates		Hipparion cf. <i>primigenium</i> (As)
Cercopithecidae		Hipparion cf. <i>afarensis</i> (MA)
Cercopithecus cf. <i>aethiops</i> (An, Ani)		Hipparion sp. (AA, SA, Bo, An)
Cercopithecus sp. ('Ha', Me)		Equus cf. <i>numidicus</i> (Et)
Papio (=Dinopithecus cf. <i>ingens</i>) (MA)		Equus sp. (Bo, Da, An)
Papio cf. <i>hamadryas</i> (Bo)		Rhinocerotidae
Theropithecus oswaldi ('Ka', MA, Bo, An)		Diceros <i>bicornis</i> ('As')
Parapapio sp. (Ka)		Ceratotherium cf. <i>praecox</i> (Ku)
cf. <i>Papionini</i> indet. (small) (Ku, Ha)		Ceratotherium cf. <i>simum</i> (MA, Bo, 'Me')
Colobus cf. <i>guereza</i> (An, Ani)		gen. et sp. indet. (AA, SA, Bo, Me, Ani)
cf. <i>Paracolobus chemeroni</i> (As, Ku)		Artiodactyla
<i>Paracolobus chemeroni</i> (MA)		Suidae
Colobinae indet. (cf. <i>Libypithecus</i>) (Ku)		Phacochoerus <i>aethiopicus</i> (Me, An, Ani)
Colobinae indet. (MA)		Kolpochoerus sp. 'A' (Ad, As)
Hominae		Kolpochoerus sp. 'B' (cf. K. 'majus') (Bo, An)
Homo sapiens cf. <i>rhodesiensis</i> (Bo)		Kolpochoerus <i>olduvaiensis</i> (Da)
Rodentia		Kolpochoerus <i>afarensis</i> (Ar/Be, MA)
Muridae		Kolpochoerus <i>limnetes</i> (MA, Bo, 'Me')
Murinae indet. (An, Ani)		Metridiochoerus <i>jacksoni</i> (MA)
Cricetidae		Metridiochoerus cf. <i>jacksoni</i> (Bo)
Gerbillinae indet. (An)		Notochoerus <i>euilus</i> (MA)
cf. <i>Dendromurinae</i> (An)		Notochoerus cf. <i>euilus</i> ('Be')
Thryonomyidae indet. (Bo)		Notochoerus <i>scotti</i> (MA)
cf. <i>Thryonomys</i> sp. (An)		Nyanzachoerus <i>kanamensis</i> (Ku, SA)
Hystriidae		Nyanzachoerus <i>jaegeri</i> (Ar/Be, Be)
cf. <i>Hystrix</i> sp. (MA, An)		Nyanzachoerus cf. <i>tulotos</i> (Ad, As)
Rodentia indet. (As, Ku, 'Ha', MA, Bo, Me, An, Ani)		Hippopotamidae
Carnivora		Hippopotamus sp. (MA, Et, Me, Da)
Canidae		Hippopotamus cf. <i>gorgops</i> (Bo)
gen. et sp. indet. (An)		Hexaprotodon cf. <i>harvardi</i> (AA, SA)
Mustelidae		Hexaprotodon sp. (small) (MA)
Lutrinae indet. (An)		Giraffidae
Felidae		Giraffa cf. <i>pygmaea</i> (MA)
Megantereon sp. (MA)		Giraffa cf. <i>jumae</i> (An)
Dinofelis sp. (MA)		Giraffa sp. (Bo)
gen. et sp. indet. (Ku)		<i>Silvatherium maurusium</i> (As, 'Ha', Ar/Be, MA)
Hyaenidae		gen. et sp. indet. (Ku)
<i>Crocota crocuta ultra</i> (MA)		Bovidae
<i>Crocota</i> sp. (An)		cf. <i>Ugandax gautieri</i> (Ku)
gen et sp. indet. ('As')		cf. <i>Pelorovis</i> sp. (Bo, An)
Carnivora indet. (Ku, Ha, Bo, An)		Bovini indet. (small) (An)
Proboscidea		Bovini indet. (large) (Me, Da, Ani)
Gomphotheriidae		Miotragoceros sp. (As)
Anancus sp. (Ad, As, Ku, Ha, Ar/Be, Be, Ka)		cf. <i>Mesembriportax acrae</i> (Ku)
Elephantidae		Boselaphini indet. (AA, Be)
Stegotetralodon cf. <i>orbis</i> (As)		?Caprini ('Me')
'Stegodibelodon schneideri' (Ad)		Tragelaphus sp. (Lothagam type) (As, Ku)
Primelephas cf. <i>gomphotheroides</i> (AA)		Tragelaphus sp. ('pre-nakuae') (Ku, Ha, 'Ka')
aff. <i>Mammuthus subplanifrons</i> (Ku)		Tragelaphus cf. <i>nukuae</i> (MA)
<i>Mammuthus subplanifrons</i> (Ar/Be)		Tragelaphus cf. <i>scriptus</i> (An, Ani)
<i>Loxodonta adaurora</i> (Ar/Be)		Tragelaphus sp. (large) (Da, An)
Elephas cf. <i>ekorensis</i> (Be, Ka)		Redunca sp. (Me, An, Ani)
Elephas <i>recki</i> (MA, Et, Bo, Me, Da, An)		Kobus cf. <i>subdolos</i> (As)
Deinotherioidea		Kobus sp. (MA)
Deinotheriidae		Kobus cf. <i>ellipsiprymnus</i> (Bo)
Deinotherium <i>bozasi</i> (SA, MA)		cf. <i>Kobus leche</i> (Bo)
Deinotherium sp. (small) (As, Ku)		Reduncini indet. (AA, MA, Ani)
		Hippotragini indet. (Ku, Ar/Be)
		Aepyceros sp. (MA)
		Aepyceros cf. <i>melampus</i> (Bo, An)
		Alcelaphus cf. <i>buselaphus</i> (Bo)
		Connochaetes sp. (Da)
		Alcelaphini indet. (Ku, Ar/Be, MA, An)
		Gazella sp. (Ar)
		cf. <i>Gazella</i> (Ku, MA, 'Me')

Formations: AA, Adu-Asa (inclusive); SA, Sagantole (inclusive); MA, Matabaietu. Sub-units: Ad, Adu; As, Asa; Ku, Kuseralee; Ha, Haradaso; Ar, Aramis; Be, Beeryada beds; Ka, Kalaloo beds; Et, Equus tuff; Bo, Bodo; Me, Meadura; Da, Dakanihyalo; An, Andalee; Ani, Andalee (Issie). Other: Provisionally assigned units in quotation marks.

The age of the Middle Awash units and their relationship to the wider African late Neogene biostratigraphical sequence can be estimated from their stratigraphical relationships and the composition of their faunas. These data are compatible with the few radiometric dates presently available from the Middle Awash. We anticipate that additional radiometric and palaeomagnetic data will provide a stronger chronological framework in the region.

The Chorora Formation is bracketed by radiometric dates of 10.5 and 9 Myr⁵, and its fauna bears resemblances to that of the Ngorora Formation¹⁵. The Fursa basalts, which underlie

the Adu-Asa Formation, are dated at 10–12 Myr¹⁶. The Lower Afar Stratoid Series (LASS) basalts², which immediately overlie the same formation, are as yet undated, but they (and the underlying Adu-Asa Formation) are clearly older than the Upper Afar Stratoid Series (UASS) basalts, whose maximum age is 4.4 Myr¹⁷. The Adu-Asa fauna is comparable with assemblages from late Miocene units in the Baringo basin—the Mpesida beds (±7 Myr), the Lukeino Formation (±6.5 Myr) and the Kaperyon Formation (±5 Myr)—and to the fauna from the Lothagam-1 beds^{18–23}. The latter is conventionally dated to ±5.5 Myr²⁰. More precise affinities of each member can be

The Wehahietu Formation is clearly Pleistocene in age. The fauna of the lower units (Equus tuff; Bodo, Meadura and Dakanihyalo Members) may be comparable with Olduvai above the Lemuta Member^{23,44}, and Ologresailie⁴⁵, while a combination of faunal, stratigraphical and archaeological evidence^{2,10} suggests that the upper Wehahietu Formation above the Andalee

Member is late Pleistocene, perhaps synchronic with the Abhe I beds of the central Afar⁴⁶. A single ¹⁴C date of 8,955±yr BP from the Holocene beds near Gewani⁴⁷ suggests that these beds are equivalent in age to lake sediments deposited in the central Afar between 5,000 and 11,000 yr BP⁴⁶.

Archaeology

We now provide a regional overview of the RVRME archaeological sites whose individual descriptions or stratigraphical contexts have been presented, or will shortly appear, elsewhere^{1,2,9,10,48–50}. An Oldowan-like industry occurs in the lower Matabaietu Formation at Matabaietu⁴⁹ and Wilti Dora, where it is associated with large mammals, especially elephants.

Table 2 Distribution of major taxa in the Awash Group[illegible]

×, Present; o, most similar or probable form present. Fm, Formation; Mbr, Member.

An early lithic site has also been reported in the upper Kada Hadar Member of the Hadar Formation⁵¹, and two of us (J.K., E.O.) have recorded a diverse Oldowan assemblage in the UHF beds. Archaeological sites in the Wehaietu Formation document middle and late stages in the development of the Acheulian Industrial Complex^{1,9,10,48,50}. In numerous localities, artefact assemblages occur in clusters in apparent primary association with the remains of single large- or medium-sized mammals, suggesting butchery sites. At other localities, the occurrence of both tools and debitage suggest tool manufacture. Tools are generally made of basalt, usually identifiable as of local derivation.

Assessment of the total early (?) to Middle Pleistocene Acheulian sequence in the lower Wehaietu Formation reveals that the Acheulian Complex is represented throughout >100 m of stratigraphical section, in an area of >25 km². From Hargufia to Subalealo alone, a distance of 20 km, individual tool occurrences are virtually continuous and number in the hundreds, with artefacts in the tens of thousands. In this area, at Bodo, and in the area surrounding Dakanihyalo and Adgabula, there are at least eight separate levels of Acheulian tools representing several distinct stages in the development of the Acheulian Complex. At all levels, tools are associated with faunal remains, often in direct context, suggesting butchery sites or living floors. At least one quarry/factory site is present (at Meadura), and the apparent occurrences of burned bone associated with tools [at Meadura (?), Adgabula] may prove to be among the earliest records for fire use known.

The MPA Gravels are associated with three distinct sets of artefacts. Within the gravels are highly rolled and weathered tools derived from older, Acheulian- (and Oldowan- ?) bearing deposits. Later artefacts occur on the surface of the gravels, derived as lag from the erosion of overlying sediments—a fact that has caused confusion about the age of this deposit^{52,53}. Corvinus⁵⁴, working at Hadar, was the first to demonstrate clearly the stratigraphical relationship of these gravels to underlying *in situ* Acheulian tool-bearing deposits. Artefacts *in situ* on the surface and, reportedly, also within the MPA Gravels⁵⁴ may bear similarities to those from Andalee.

Artefacts from the lower sequence at Andalee represent one of the rare occurrences of an *in situ* 'Sangoan' industry and are associated primarily with very small- to medium-sized mammals¹⁰. Finally, the assemblage from Issie contains a diversity of retouched, well-formed tools that suggest later industrial influences. Middle Stone Age tools predominate in assemblages elsewhere in the upper Wehaietu Formation. Late Stone Age microlithic tools made from silicic materials occur throughout the Holocene beds, especially at Nemay Koma and Mero Buri. Ceramics occur at several localities on or adjacent to the Awash floodplain.

Discussion

When added to the previously-described Hadar Formation, the stratigraphical sequence running from the Adu-Asa Formation through the Wehaietu Formation provides one of the longest and most complete records of land vertebrate evolution yet recognized in the African Neogene. In particular, the formations and faunas newly described here document faunal evolution immediately preceding the well-known middle Pliocene of Hadar, and provide a uniquely continuous record of faunal, cultural and environmental change during the last half of the Pleistocene.

The faunas of the latest Miocene to earliest Pliocene and Middle to late Pleistocene of Africa are known from scattered sites that in most cases cannot be unambiguously arrayed against a single stratigraphical and chronological scale. Hopefully such a scale should be significantly improved now that the essentials of the stratigraphy of the Middle Awash deposits are known, intercalated as they are with volcanic materials. The collections reported here have already contributed to knowledge of the history of major taxa during this period. Among the Bovidae,

for example, are a combination of genera (*Miotragocerus*, *Mesembriportax*, *Ugandax*) known separately from North, South and East Africa²⁴. Among the Suidae, the succession of species in the genus *Nyanzachoerus* is documented, as is that for *Kolpochoerus*. The latter includes what may be the earliest African suine in the Adu and Asa Members, a primitive *Sus*-like form which tends to confirm Cooke's hypothesis about the origins of this genus⁵⁵. Advanced kolpochoeres in the Wehaietu Formation provide insights into what may be among the latest representatives of this genus.

The Proboscidea include material that documents extensively some hitherto poorly known taxa. The gomphotheriid *Anancus* occurs throughout the Adu-Asa and Sagantole Formations, throughout >350 m of section. This occurrence is more extensive than any previously described from trans-Saharan Africa, and will ultimately form the basis of a full revision of the group, one of major biostratigraphical importance. At present, the Awash anancines are grouped into evolutionary stages, in the course of which molars become larger and more complex. Stages in the series serve to link sites such as Langebaanweg, Sahabi and Lothagam. Similarly, all recognized genera of Elephantidae are present in the Middle Awash. These include stegotetrabelodonts, as well as a variety of true elephantines that particularly document early stages of diversification of this subfamily. The biogeographical implications of the presence of elephantines in the late Miocene of East Africa, and their absence from the post-Messinian of Libya, are discussed elsewhere⁸.

At least seven cercopithecoid genera are recognized in the Middle Awash. Those accorded the most attention, species close to the modern *Cercopithecus* and *Colobus* monkeys, are exceptionally well-represented in the Andalee Member¹⁰. The excellent preservation and abundance of monkeys of these forms and age are unknown elsewhere. *Theropithecus* (*Simopithecus*) is also abundantly represented, in almost all known forms of *T. oswaldi*⁵⁶ (including, as a subspecies, *T. darti*). These range from a primitive form from Wee-ee (Kalaloo beds?), similar to material known throughout the Hadar Formation, to derived forms from the Wehaietu Formation that recall *T. o. mariae* and *T. o. leakeyi* from East Africa⁵⁶.

The recognition of extensive, highly fossiliferous strata antedating the Hadar Formation raises the possibility of documenting the earlier stages of hominid evolution. The hominid remains from the Hadar Formation have been vigorously touted as representatives of a single, thoroughly plesiomorphic hominid species ancestral to all known later forms³⁷. This view is not unchallenged, others (see, for example, ref. 57) seeing representatives of two lineages in the collection. In any case, there is evidence that bipedal hominids were present earlier than 4 Myr at Kanapoi⁵⁸, and a hominid-like hominoid of uncertain affinities and unknown locomotor type is known from Lothagam-1⁵⁹. It is hoped that the Adu-Asa Formation, which contains abundant fauna and apparently overlaps both Lothagam-1 and Kanapoi, will in time yield hominid specimens sufficiently complete to be assignable to an unambiguous position within the hominid clade, and thus to throw light on the timing and circumstances of the initial radiation of the family.

Like other East African sites, the Middle Awash sees the first appearance of clearly recognizable human artefacts in the late Pliocene (Matabaietu and upper Hadar Formations). When analysed more fully, the many occurrences of artefacts together with the remains of animals in this and the Wehaietu Formation will permit the testing of hypotheses about the relative importance of hunting, scavenging and fortuitous association in producing such archaeological sites in the Plio-Pleistocene. The hominid skull recovered from the base of the Wehaietu Formation at Bodo¹¹ not only provides critical evidence on archaic *Homo sapiens*; it also provides an unequivocal relationship between the morphological grade of tool-maker with a specific 'grade' in Acheulian technology. Overlying Acheulian tool-bearing units may yield other hominids of potentially more derived forms. Most significantly, the presence of ample tuffs

throughout the Acheulian sequences allows for construction of a chronometric framework for both changing technologies and technicians, in what may well be the single most extensive Acheulian deposits described.

The diversified lithic 'Sangoan' industry at Andalee may document that transitional period between the longstanding conservatism of the Acheulian and the relative cultural diversity that characterizes the late Pleistocene⁷. The Andalee industry and its predominantly smaller fauna stand in marked contrast to the association of large tools and large game animals found in the Acheulian sites. Kalb *et al.*⁹ speculate this may reflect adjustments to faunal turnover and changing man-land relationships following a prolonged arid period at the end of the Middle Pleistocene. Further studies at Andalee will elucidate this question, as will work at nearby Issie.

Overall, the succession of Acheulian, Sangoan and MSA industries in the Wehaietu Formation may provide a reasonable picture of man-land relationships during the middle and late Pleistocene during such crucial events as the first appearance of artificial fire in the region, the Acheulian-Sangoan transition, and thence to the MSA and LSA, and the appearance of modern humankind.

Conclusions

The Middle Awash Valley contains a largely complete record of mammalian evolution spanning at minimum the past 5–6 Myr. This record is contained within a stratigraphical sequence >1 km thick, making it one of the most extensive fossil sequences in the African Neogene. Evidence of hominid habitation, in the form of hominid fossils or artefacts, is found here in >0.5 km of beds, extending over ~4 Myr, from the lower levels of the Hadar Formation³⁷ to the Matabaietu Formation, through the Wehaietu Formation and the Holocene beds. The Middle Awash contains one of the most continuous

records of hominid habitation in the Old World. Likewise, the stone tool occurrences extending throughout over 300 m of the Awash sequence, encompassing of the order of 2–2.5 Myr, from the upper Hadar Formation and the Matabaietu Formation to the Recent, documents one of the most continuous records of human cultural change known.

The present work and that in our previous paper, should lay a firm foundation for a coherent and orderly programme of research in the Middle Awash in the years to come.

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Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence

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Chemically synthesized peptides corresponding to two different regions of the VP1 polypeptide of foot-and-mouth disease virus (FMDV) produced high levels of serotype-specific virus neutralizing antibody in cattle, guinea pigs and rabbits. A single inoculation of one of these peptides protected guinea pigs against subsequent challenge with the virulent virus.

FOR at least four centuries foot-and-mouth disease has been one of the world's major animal plagues¹. The virus affects both domestic and wild ruminants and swine. Its ease of dissemination, coupled with the loss of productivity of the animals following infection, usually estimated at 25%, make it a disease of major concern. The problem is worldwide as the disease is present in every continent except Australia and North America. The disease is controlled by slaughter in those countries where it does not normally occur but vaccination is used where it is endemic. The extent of the problem is such that over 1,000 million doses of vaccine are used each year but, although vaccines have been available for the past 40 years, the disease is still widespread. The vaccines are produced by inactivation of virus harvests produced in cultures of baby hamster kidney cells or in bovine tongue epithelium fragments. Although these vaccines are generally effective, there are certain drawbacks to their use. For example, several strains of the virus cannot be grown to a titre high enough to provide sufficient antigenic mass for effective vaccination. In addition, the virus particle is very unstable, particularly below pH 7, so it is necessary to store vaccines under refrigeration to retain their potency. This is clearly a major limitation, particularly in tropical climates. The recent association of outbreaks of the disease in Europe with improperly inactivated virus² illustrates another disadvantage of this type of vaccine, particularly in those regions of the world where the disease has been virtually eradicated. These disadvantages have prompted continuing studies on the structural features of the virus particle that are necessary for immunogenic activity with a view to developing alternative vaccines.

The virus, which belongs to the aphthovirus genus of the family Picornaviridae, contains one molecule of infectious single-stranded RNA of molecular weight (M_r) $\sim 2.6 \times 10^6$ and 60 copies of each of four polypeptides VP1–VP4 (ref 3). An important antigenic role for VP1 is indicated by the fact that treatment of the virus particle with trypsin results in the cleavage of only this polypeptide, with a concomitant decrease in infectivity and, depending on the virus strain, a loss of immunizing activity⁴. Indeed, VP1 isolated from virus particles has some, albeit very low, immunizing activity^{5–8}, whereas the other three viral polypeptides have no such activity. Based on this observa-

Table 1 Antibody response of rabbits to different peptides of VP1 of FMDV, type 0, strain Kaufbeuren

Rabbit no.	Peptide	Anti-peptide antibody titre	Neutralization index (log ₁₀)*
1	9–24	80–160	≈ 0.3
2	9–24	40–80	≈ 0.3
3	17–32	80–160	≈ 0.5
4	17–32	20–40	≈ 0.9
5	25–41	40–80	≈ 0.5
6	25–41	640–1280	≈ 0.9
7	1–41	320–640	≈ 0.9
8	1–41	320–640	≈ 0.7
9	141–160	320–640	≈ 3.9
10	141–160	320–640	≈ 3.7
11	151–160	80–160	2.9
12	151–160	160–320	1.1
13	200–213	>1280	3.5
14	200–213	160–320	3.1

In the second column, numbers refer to the amino acid, starting at the amino-terminus. Rabbits were immunized with peptide-coupled KLH inoculating 200 μ g in complete Freund's adjuvant (1:2) s.c. in the footpad on day 0; 200 μ g in incomplete Freund's adjuvant s.c. on day 14; 200 μ g with 4 mg of aluminium hydroxide intraperitoneally (i.p.) on day 21 and day 50 and were then bled on day 57. The enzyme-linked immunosorbent assay (ELISA) was used to test all antisera for their ability to react with the peptide used for immunization. Antibody was bound to antigen immobilized on polystyrene plates and the antigen-antibody complex was detected using an enzyme-labelled goat anti-rabbit IgG. Five pmol of antigen in 0.1% bovine serum albumin (BSA) phosphate-buffered saline (PBS) were added to each well of a 96-well microtitre plate and dried overnight at 37°C. The dried antigen was then fixed to the plates with 50 μ l per well of methanol for 5 min at room temperature. Before adding the antibodies, the plates were coated for 4 h with 3% BSA/PBS to block nonspecific adsorption of antibodies to the plate. Twofold serial dilutions of each antiserum were prepared in minimal essential medium containing 10% fetal calf serum, 25 μ l of the serum dilution were added per well and the plates were incubated overnight at room temperature. Unbound antibody was then washed off with water. The antigen-antibody complex was reacted with 25 μ l per well of 1:100 dilution of goat anti-rabbit IgG coupled to glucose oxidase. Excess goat anti-rabbit antibody was washed off with water and the reaction was developed by adding 50 μ l per well of developing solution [25 ml 0.1 M Na-phosphate buffer, pH 6.0, 3 ml 20% glucose, 200 μ l 0.1% horseradish peroxidase, 200 μ l of chromogen (2,2'-amino-di-[3-ethylbenzthiazolin-sulphonate]) at 20 mg ml⁻¹ in Na-phosphate buffer, pH 6.0]. The plates were read in a Titertek Multiskan at 414 nm, and the values presented are the reciprocal of the serum dilution binding to 50% of 5 pmol of antigen.

* Equal volumes of virus dilutions and undiluted sera were inoculated i.p. into suckling mice. The values give the difference between the titre of the virus plus normal serum and the virus plus antiserum.

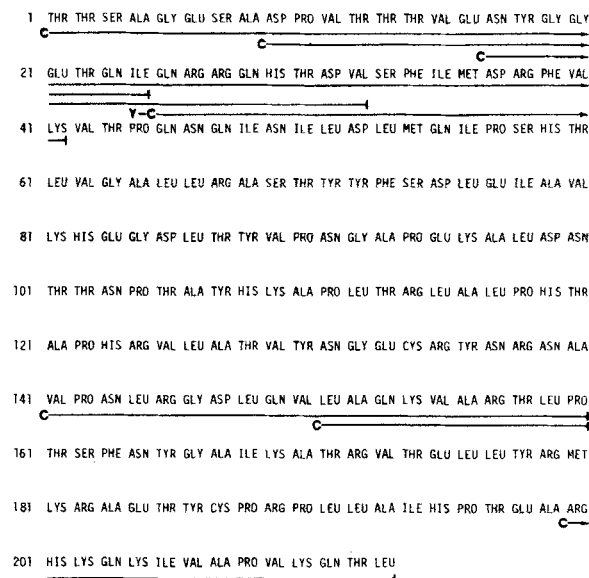


Fig. 1 The 213-amino acid sequence of VP1 as translated by Kurz *et al.*¹³ from the nucleic acid sequence. Regions of the protein chosen for synthesis are indicated by bold underlining. C or Y at the end of such a line indicates the addition of cysteine or tyrosine not found in the primary sequence. Peptides were synthesized by the solid phase method¹⁷⁻¹⁹ using a Beckman Model 990B peptide synthesizer. The initial amino acid resin was prepared by esterification of Boc-(MeoBzl)-Cys-OH (2.0 g of the anhydrous caesium salt, 4.3 mequiv) to chloromethylated resin (polystyrene-1% divinylbenzene, bio-beads S.X.-1, 200-400 mesh, 0.70 mmol g⁻¹) in 25 ml anhydrous dimethylformamide (DMF) by stirring for 24 h at 50 °C. A threefold excess of caesium salt over active chloride polymer was found to give almost complete substitution. Substitution was determined to be 0.55 mequiv g⁻¹ by the picric acid test and amino acid analysis, and 0.53 mequiv g⁻¹ following HCl-propionic acid hydrolysis. For each synthesis, 1.0 g (0.53 mequiv) of Boc-(MeoBzl)-Cys resin was used. The following side-chain protecting groups were used: *O*-Br-Z for lysine and tyrosine; *O*-benzyl for threonine, serine, aspartic acid and glutamic acid; tosyl for arginine; *N*-formyl for tryptophan; and *N*-dinitrophenyl for histidine. Protected amino acids (from Vega Biochemicals or Peninsula Laboratories) were recrystallized from the appropriate solvent to give single spots on TLC (chloroform/acetic acid = 15:1 or chloroform/methanol = 10:1). All couplings were carried out using a 10-fold excess of Boc-AA-OH. For asparagine and glutamine, an equimolar amount of *N*-hydroxy benzotriazole was added to the protected amino acid and dimethyl formamide was used as the solvent. All coupling reactions were $\geq 99\%$ complete by the picric acid test. The removal of dinitrophenyl from histidine-containing peptides was accomplished with *N*-methyl mercaptoacetamide²⁰ in DMF by mixing with the resin-bound peptide for 15 min. Removal was $>95\%$. The protected peptide polymers (500 mg to 2.5 g, 0.12-0.60 mequiv) were treated with twice their weight of anisole and with 40 times their volume (relative to weight) of anhydrous HF at 4 °C for 1 h. For peptides containing glutamic acid, 5% v/v pyridine was added to the HF to prevent anisylation. After evaporating the HF with a stream of N₂, the red residue was mixed with anhydrous ether (50 ×, w/v) and stirred vigorously for 15 min; after repeating the ether extraction three times, the white residue was filtered and dried *in vacuo*. This dried resin-peptide mixture was generally extracted with 5% HOAc, lyophilized and amino acid analysis performed (approximately 90% removal of the peptide from the resin was accomplished in this manner). For peptides containing tryptophan, the *N*-formyl protecting group was removed from the peptide by treatment for 1.5 min at pH 11.5 (NaOH) in the presence of hydrazine. If the amino acid analysis was acceptable ($\pm 5\%$ of theory), the peptides prepared in the above manner were used directly; if not, they were purified to the desired extent by CM-cellulose chromatography, partition chromatography, or HPLC as needed. Yields ranged from 20-60% depending on the given peptide, and the purity needed. The peptides were coupled to a protein carrier, KLH, through the cysteine of the peptide, using *N*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as a coupling agent, as described by Liu *et al.*²¹.

Application of recombinant DNA technology has also allowed nucleotide sequences of the virus genome to be determined, from which the primary amino acid sequence of VP1 can be deduced. Sequences are now available for two strains of serotype A^{11,12} and one of type O¹³. In addition, Strohmaier and his colleagues¹⁴ have provided evidence, by measuring the immunogenic activity of enzymatically or chemically derived fragments of VP1 of the type O strain, that sites eliciting neutralizing antibody are located in the carboxy-terminal half of the molecule. We have chemically synthesized peptides corresponding to several regions of VP1 of this strain of virus and report here that some of these induce neutralizing and protective antibodies against the disease.

Chemically synthesized peptides induce neutralizing antibodies in rabbits

Previous studies¹⁴ indicated that the carboxy-terminal region was important in inducing neutralizing antibody. In addition, nucleic acid sequencing of the two strains of type A^{11,12} and one of type O¹³ demonstrated that there was considerable variability between positions 130-160 and 190-213. We reasoned that this variability was due to the availability of these regions for interaction with neutralizing antibody and their consequent subjection to high selective pressure. To study the molecule further, we also selected a series of N-terminal peptides for synthesis. Seven peptides from VP1 of type O virus (strain Kaufbeuren), corresponding to four regions near the amino-terminus (1-41), one at the carboxy-terminus (200-213) and two near the centre of the molecule (141-160 and 151-160) were synthesized, coupled to keyhole limpet haemocyanin (KLH) and inoculated into rabbits (see Fig. 1). The KLH-coupled peptides elicited anti-peptide antibody titres as assayed by ELISA (Table 1). However, a virus neutralizing antibody response was elicited only by the peptides in the middle region and at the carboxy-terminus.

The serotype specificity of the antibodies produced in the rabbits by the peptides spanning regions 141-160 and 200-213 was determined in neutralization tests using the homologous virus and viruses of types A and C. Although there was some cross-neutralization, the level was considerably lower than that of the homotypic reaction (Table 2). These results show that the serotype specificity of the sera produced by inoculation of the synthetic peptides mimicks that found with sera against whole virus. The cross-neutralization may reflect the limited sequence homology among the different serotypes.

Guinea pigs immunized with synthetic peptides are protected against foot-and-mouth disease

Having established that peptides 141-160 and 200-213 produced high levels of neutralizing antibodies on multiple inoculation into rabbits, we tested the effectiveness of a single inoculation of the antigens in producing neutralizing antibodies in guinea pigs and in providing protection against challenge. Guinea pigs were inoculated subcutaneously (s.c.) with 20 or 200 µg of either of the two peptide conjugates, mixed with

Table 2 Serotype specificity of the anti-peptide antibodies

Antibody to peptide region:	Neutralization index (log ₁₀)* against		
	0 Kaufbeuren	C Indaial	A-61
141-160 (rabbit 9)	4.3	-0.1	1.1
141-160 (rabbit 10)	≥ 6.3	1.9	2.3
200-213 (rabbit 13)	2.9	-0.1	1.5
200-213 (rabbit 14)	3.3	0.3	1.5

C Indaial belongs to serotype C, subtype 3; A-61 belongs to serotype A, subtype 10.

* See Table 1 legend.

tion, workers in Europe and the USA⁹⁻¹¹ have investigated the possibility of producing the protein by recombinant DNA technology, and Kleid *et al.*¹¹ have produced by this means $1-2 \times 10^6$ copies of VP1 per *Escherichia coli* cell. However, the expressed protein appears to be no more active weight for weight than VP1 produced from virus particles and it is clear that much remains to be learned about the structural features necessary for effective immunization before VP1 can be considered a realistic alternative to killed virus particles.

Freund's complete adjuvant or aluminium hydroxide gel. Parallel groups of animals were inoculated with either 1 or 10 μg of purified inactivated virus particles mixed with either of the same two adjuvants. After 35 days, the animals were bled by heart puncture to determine the level of neutralizing antibody and then challenged by inoculating one hind footpad with 10^4 ID₅₀ (50% infectious dose) of the homologous virus which had been passaged once in guinea pigs. The results, summarized in Table 3, demonstrate the effectiveness of peptide 141–160 in producing neutralizing antibody and protecting the animals against infection. As little as 200 μg provided complete protection and even 20 μg provided substantial protection, in view of the severity of the challenge. There was good correlation between the level of neutralizing antibody and the response to challenge. Those animals whose sera had a neutralizing index of ~ 1.5 or greater were protected.

In contrast, inoculation of guinea pigs with a similar amount of VP1, prepared by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gave a much lower neutralizing antibody response. We have observed this for several different preparations of VP1 from viruses of types 0 and A.

Biochemical characterization of the virus components reacting with the anti-peptide sera

The rather surprisingly superior neutralizing antibody response of the animals to inoculation with the synthetic peptides, compared with that observed in studies using VP1, prompted us to explore the basis for this difference. As only the antisera to peptides 141–160 and 200–213 had neutralizing activity, we confined our experiments to these and we have characterized the reactivities of these novel reagents in a variety of assays commonly used in this virus system. First, the antisera were tested for their ability to precipitate purified ³⁵S-methionine-labelled virus antigens (Table 4). Intact 146S virus particles were precipitated by (1) the anti-peptide sera, (2) a rabbit antiserum prepared against purified VP1, (3) convalescent guinea pig serum and (4) guinea pig serum prepared against 146S particles. With the exception of the serum against the 146S particles, the same antisera also precipitated the 12S virus protein subunit, which is a pentamer of VP1–3, produced by heating 146S particles to 60°C. The lack of reaction between antisera to 146S particles and the 12S subunit has been described previously¹⁵. Disruption of the 146S particle into its constituent polypeptides by treatment with 8M urea at 37°C, followed by dilution to reduce the urea concentration, led to a loss of reactivity with the anti-146S serum and a considerable reduction in its reaction with the convalescent serum. However, there was less reduction with three of the four anti-peptide sera. The anti-VP1 serum precipitated the denatured protein efficiently.

Table 3 Protection of guinea pigs against challenge with FMDV by inoculation with inactivated virus particles, VP1 or synthetic peptides 141–160 and 200–213

Antigen	Dose (μg)	Adjuvant	Neutralization index (log ₁₀)*	No. protected/ No. challenged
Virus	1	Al(OH) ₃	2.7(1.1–2.3)	1/4
	10	Al(OH) ₃	$\geq 4.9(3.4–\geq 4.5)$	2/2
	1	Freund's	2.5(1.3–2.9)	2/2
	10	Freund's	$\geq 5.3(\geq 4.5)$	4/4
VP1	20	Al(OH) ₃	0.5(0.1–0.9)	ND
	20	Al(OH) ₃	2.1(0.9–2.7)	3/4
	200	Al(OH) ₃	2.7(1.7– ≥ 3.7)	3/3
	20	Freund's	2.1(0.1–2.5)	1/4
141–160	200	Freund's	$\geq 3.3(1.5–\geq 3.3)$	4/4
	20	Al(OH) ₃	1.1(ND)	1/3
	200	Al(OH) ₃	0.7(ND)	2/4
	20	Freund's	1.1(ND)	0/4
200–213	200	Freund's	0.5(ND)	0/4

* Neutralizing activity of pooled serum from eight animals. Values in parentheses give the range of activity in the individual animals that were challenged. ND, not done.

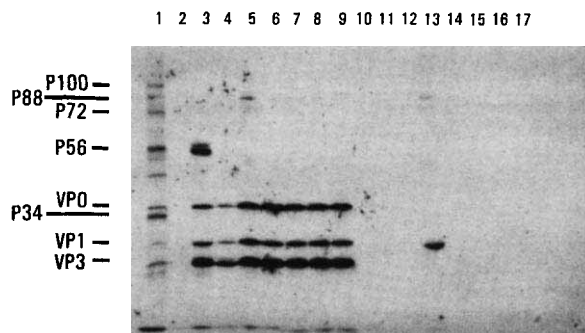


Fig. 2 Comparison of immune precipitation of viral proteins by different antisera. FMDV-infected BHK 21 cells were labelled with ³⁵S-methionine from 2.5 to 3 h post-infection, when host cell protein synthesis was almost completely halted. The cells were then lysed by treatment with 1 ml of 1% NP-40, 0.5% DOC in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5 and the nuclei pelleted in a microfuge for 5 min. Aliquots (20 μl) of the supernatant were diluted with 1 ml of NETS (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5 and 0.5% NP40) immediately, or after adding SDS to 1% and heating at 100°C for 2 min. The appropriate antiserum (30 μl) was added to each sample and complexes allowed to form at 4°C overnight. Washed, inactivated *Staphylococcus aureus* ghosts (Pansorbin) were then added to adsorb IgG molecules and separated by centrifuging for 5 min in a microfuge. The pellets were washed twice with NETS and the adsorbed IgG and complexed antigens eluted by suspending the pellets in Laemmli disruption buffer²². The proteins were denatured by heating at 100°C for 2 min and separated on an 11% Laemmli gel²². The gels were prepared for fluorography²³ and exposed to X-ray film. Lane 1, total cell extract; lanes 2–9, immune precipitates of undenatured extracts; lanes 10–17, immune precipitates of SDS-denatured extracts. Lanes 2 and 10, control rabbit serum; lanes 3 and 11, convalescent guinea pig antiserum; lanes 4 and 12, guinea pig antiserum to 146S particles; lanes 5 and 13, rabbit antiserum to VP1; lanes 6 and 14, antiserum to peptide 141–160; lanes 7 and 15, antiserum to peptide 141–160; lanes 8 and 16, antiserum to peptide 200–213; lanes 9 and 17, antiserum to peptide 200–213.

The same antisera were then tested for their ability to precipitate the virus-specific antigens in infected BHK 21 cells. The antigens were extracted in their native state by disrupting the cells with deoxycholate (DOC) and Nonidet P-40 (NP40)¹⁶ and used either directly or after denaturation by heating at 100°C in the presence of 1% SDS. The immunoprecipitated proteins were separated by PAGE (Fig. 2); all the antisera precipitated both the virus structural proteins and their precursor, P88 (lanes 3–9). The convalescent antiserum also precipitated some of the non-structural polypeptides. As the structural proteins are present as complexes when extracted from cells in these conditions, antisera reactive with VP1 also precipitate the other structural polypeptides.

After denaturation of the proteins with SDS, only the rabbit anti-VP1 serum precipitated VP1 and the structural protein precursor P88 (Fig. 2, lane 13); VP1 was precipitated neither by the control rabbit serum (lane 10), by convalescent serum (lane 11) nor by the anti-146S serum (lane 12). The antisera against peptides 141–160 (lanes 14, 15) and 200–213 (lanes 16, 17) precipitated very little of the SDS-denatured antigens; a very faint VP1 band could be seen in the 200–213 precipitates on the original autoradiograph.

The reactivity of the various antisera for denatured molecules was further examined by the Western blotting technique, whereby the proteins from purified denatured virus particles were separated by SDS-PAGE, transferred to nitrocellulose, probed with antiserum and the reaction visualized by autoradiography after binding ¹²⁵I-labelled protein A. The results (Fig. 3) again show a lack of reaction between VP1 and the convalescent and anti-146S sera (lanes 2 and 3) and a strong reaction with anti-VP1 serum (lane 4). Reaction between the 200–213 antisera and VP1 (lanes 7, 8) was more obvious than

in the immunoprecipitation experiment (Fig. 2); only a weak reaction with the two anti-141-160 sera (lanes 5, 6) was seen.

Taken together, these data indicate that the anti-peptide sera react with the protein species containing the appropriate sequence, including precursor molecules, and that they resemble anti-viral anti-sera in reacting well with native components. Because of their propensity to bind to native VP1, these sera also precipitate other protein species which, in non-denaturing conditions, are bound to the primary target by virtue of the protein-protein interactions important in the structure of this virus. On the other hand, it is clear that the activity of the anti-VP1 serum is directed primarily against denatured protein, an observation easily understandable in view of the fact that this serum was prepared against SDS-denatured protein. VP1 isolated by other means has been an equally poor antigen for generating an anti-viral response (D.J.R. and F.B., unpublished work).

Discussion

Our results show that a single inoculation of the synthetic peptide 141-160 elicits sufficient neutralizing antibody to protect guinea pigs against subsequent challenge with FMDV. Although we have not yet accumulated sufficient data to allow a precise comparison, the results obtained so far indicate that, on an equal weight basis, a single peptide has from one to as much as 10 per cent of the activity of the inactivated virus particle. Furthermore, the neutralizing antibody titre elicited

Table 4 Precipitating activity of different antisera against the 146S virus particle, 12S subunit and viral polypeptides

Serum	Animal	Amount of antigen precipitated (counts per 20 min)		
		146S particle	12S subunit	Viral polypeptides
Normal	Rabbit	667	616	637
Convalescent	Guinea pig	2,692	1,833	742
146S particle	Guinea pig	2,889	668	646
VP1	Rabbit	3,231	2,457	1,553
141-160	Rabbit 9	3,123	2,212	774
141-160	Rabbit 10	3,174	2,312	1,047
200-213	Rabbit 13	3,434	2,259	1,070
200-213	Rabbit 14	3,215	2,266	1,127

by the peptide is several orders of magnitude greater than the best results obtained by immunization with the capsid protein VP1, irrespective of whether this is produced by disruption of virus particles⁵⁻⁸ or by expression in *E. coli* cells¹¹. Indeed, in several of our own experiments (for example, see Table 3) and in other studies⁵⁻⁸, the level of neutralizing antibody response to one inoculation of the capsid protein was negligible. The reason for this difference may be that the antibody induced by the synthetic peptide is more closely related qualitatively to that induced by virus particles than to that induced by VP1. It

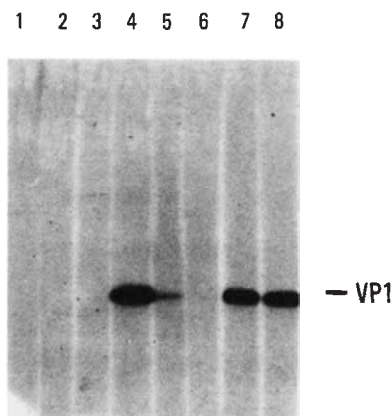


Fig. 3 Comparison of the reaction of different antisera with separated viral polypeptides. Purified FMDV 146S particles were denatured with Laemmli disruption buffer²² at 100 °C for 2 min and the proteins separated on a 10% polyacrylamide gel slab. The proteins were electrophoretically transferred to a nitrocellulose sheet which was then cut into strips. The strips were immersed in a solution of 10 mg ml⁻¹ haemoglobin containing the different antisera. After thorough washing, the strips were immersed in a solution of ¹²⁵I-labelled *S. aureus* protein A. The washed, dried strips were then exposed to X-ray film to visualize the binding of protein A to IgG molecules immobilized on the strips. Lane 1, control rabbit serum; lane 2 convalescent guinea pig antiserum; lane 3, guinea pig antiserum to the 146S particle; lane 4, rabbit antiserum to VP1; lanes 5 and 6, rabbit antisera 9 and 10 to peptide 141-160; lanes 7 and 8, rabbit antisera 13 and 14 to peptide 201-213.

is possible that the separated VP1 does not fold properly when deprived of the scaffolding provided by the other capsid proteins. Thus the immunodominant site presented to the immune system by free VP1 may be largely irrelevant to neutralization. In contrast, a small free peptide may be able to adopt a conformation approximating that which it assumes in the virus particle, a situation that is unlikely when it is constrained by the neighbouring amino acids in an improperly folded VP1.

One clear advantage of the synthetic 141-160 peptide is its activity in eliciting a protective antibody response after a single inoculation. This good response to a single inoculation is important, because successful immunization against foot-and-mouth disease in the field depends on the vaccines being sufficiently active to produce a protective response with one inoculation. Indeed, preliminary work in cattle and pigs shows that the synthetic peptide 141-160 can elicit an antibody response sufficient to protect these species against the disease. Thus the prospects for a totally synthetic vaccine against foot-and-mouth disease are encouraging.

We thank David Morrell for the Western blot experiment and Janet Haresnape for the anti-VP1 serum.

Note added in proof: Since the submission of this article, we have obtained evidence that synthetic peptides corresponding to amino acids 141-160 of the VP1 of two subtypes of serotype A of FMDV (subtype 10, strain 61 and subtype 12, strain 119) also elicit the production in rabbits of levels of neutralizing antibody similar to those described here.

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Transcript secondary structures regulate transcription termination at the attenuator of *S. marcescens* tryptophan operon

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We have analysed the regulatory behaviour of deletion mutants lacking different segments of the leader region of the tryptophan operon of Serratia marcescens. Our results support the model in which a particular RNA structure, the terminator, is recognized during transcription as a transcription termination signal, and an alternative RNA structure, the anti-terminator, prevents formation of the terminator. It appears that the role of translation, ribosome stalling and shifts between alternative RNA secondary structures, is simply to regulate formation of the terminator.

SEVERAL bacterial operons encoding amino acid biosynthetic enzymes are regulated by attenuation (for reviews see refs 1–3). This regulatory mechanism involves controlled transcription termination at a site, the attenuator, located in the leader region, the segment of the operon between the transcription start site and the first structural gene. Comparison of the sequences of the leader regions of the six-amino acid biosynthetic operons known to be regulated by attenuation^{4–7} reveals considerable similarities. Each operon has a potential peptide-coding region in the leader transcript which, if used, would specify a small peptide rich in the regulatory amino acid. Immediately beyond, there is a site of transcription termination, an attenuator. This site consists of an A + T-rich sequence preceded by a G + C-rich sequence exhibiting dyad symmetry. The segment of the transcript corresponding to the G + C-rich region in each operon can potentially form a stable hydrogen-bonded stem and loop structure. Transcription termination at the attenuator of each of these operons is controlled by the extent of charging of appropriate transfer RNAs with the regulatory amino acid, implicating leader peptide synthesis in the regulation of termination.

A model attempting to explain attenuation was proposed by Lee and Yanofsky⁴ for the *trp* (tryptophan) operon of *Escherichia coli*. It was suggested that a G + C-rich secondary structure forms in the transcript just before RNA synthesis stops and serves as the recognition signal for transcription termination at the attenuator. An alternative secondary structure in the transcript was also predicted. If this structure, located in the earlier segment of the leader transcript, formed, it could prevent formation of the presumed transcription termination signal. It was hypothesized that this alternative secondary structure would eliminate transcription termination at the attenuator and thereby increase expression of the operon. The switch between the alternative RNA secondary structures was proposed to be regulated by ribosome movement along the transcript during translation of the coding region for the leader peptide. Specifically, ribosome stalling at the tandem Trp codons of the transcript—a consequence of the unavailability of charged tRNA^{Trp}—was thought to mask a critical segment of the transcript, thus favouring formation of the RNA stem and loop mutually exclusive with the formation of the termination secondary structure. This interpretation is consistent with the results of subsequent studies on attenuation performed with the *trp* operons of *E. coli* and other enteric bacteria^{4–7}. The model has been applied to other amino acid biosynthetic operons; it has been used to explain attenuation in the *phe*⁸, *his*⁹, *thr*¹⁰, *leu*¹¹ and *ilv*^{12,13} operons.

Many features of the proposed model have not been verified by studies with any of the operons mentioned. We report here the results of experiments designed to test the fundamental hypothesis that alternative RNA secondary structures are involved in the regulation of transcription termination at the *trp* attenuator.

A model for attenuation in the *trp* operon of *S. marcescens*

Mutational and physiological studies with *Serratia marcescens* indicate that the *trp* genes of this enterobacterium are coordinately regulated in response to changes in tryptophan concentration^{14–16}. The regulatory region of the *S. marcescens trp* operon is organized identically to those of *Escherichia coli* and *Salmonella typhimurium*¹⁷. There is a promoter-operator used to regulate transcription initiation and a leader region within which transcription termination is controlled.

Figure 1 gives the alternative RNA secondary structures of *S. marcescens* analogous to those of the *E. coli* leader transcript, and Fig. 1a shows the secondary structures that could be predicted to form *in vitro*. On the basis of studies with the *trp* operon of *E. coli*, the *in vivo* studies described here, and the results of *in vitro* transcription experiments to be presented elsewhere, we believe that the stem and loop structure labelled 3:4 in the figures is the structure recognized by the transcribing polymerase as a transcription termination signal; for example, deletion mutants that can only form structure 3:4 efficiently terminate transcription at the attenuator *in vivo* and *in vitro*. In view of these findings, structure 3:4 will henceforth be designated the 'terminator'¹¹. When cells are starved of tryptophan, the ribosome translating the peptide-coding region is believed to stall over either of the adjacent Trp codons, thereby masking transcript segments A1, 1B and 2B. In these conditions, secondary structure 2A:3 could form before the synthesis of segment 4, and thereby preclude formation of the terminator. Substantial *in vivo* and *in vitro* evidence indicates that structure 2A:3 has an important role in preventing termination. We shall designate a structure such as 2A:3 in Fig. 1b as an anti-terminator. The stalled ribosomes shown in Fig. 1b are centred over the His and Arg codons of the peptide-coding region. Ribosomes stalled at these positions would block pairing of 1A with 2A, and 1B with 2B, but would not interfere with pairing of 2A with 3. These schematic representations are based on the findings in Table 1 showing that starvation for His, Trp or Arg relieves transcription termination at the *trp* attenuator of *S. marcescens*. Starvation for proline, a residue not present in the leader peptide, has no effect. These observations support the conclusion derived from comparable studies performed with *E. coli*⁶ and suggest that attenuation is regulated through ribo-

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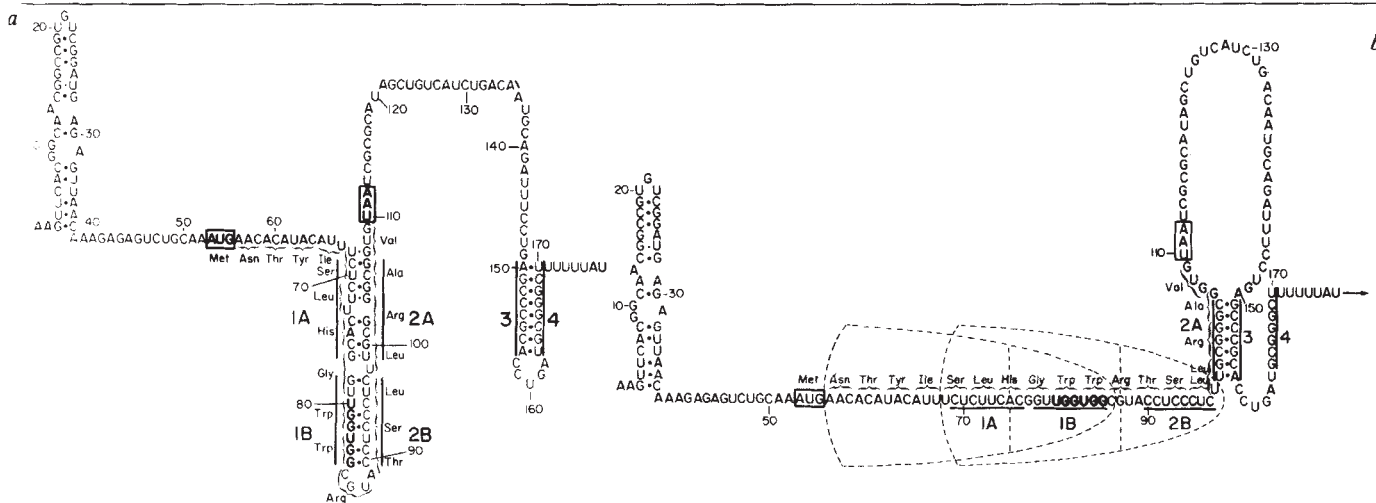


Fig. 1 . Secondary structure alternatives in the *S. marcescens* *trp* leader transcript. Numbering is from the presumed 5' nucleotide of the transcript. The likely leader peptide initiation and termination codons are enclosed in boxes and the predicted amino acid sequence of the transcript is as reported¹⁷ except for an additional A residue at position 150. The numbered RNA segments are those thought to participate in the various secondary structures that either cause or prevent transcription termination at the attenuator. The structures in *a* are predicted to form *in vitro*; hairpin loop 3:4 is believed to be the signal responsible for transcription termination. When the transcript is translated *in vivo* coincident with transcription of the *trp* leader region, structure 3:4 is thought to form and cause transcription termination. *b* Shows an alternative secondary structure. This structure, 2A:3, is believed to form in cells starved of His, Gly, Trp or Arg because the translating ribosome (schematically represented as dashed bullets stalled over His and Arg codons) stalls in the critical region of the transcript. Formation of structure 2A:3 prevents formation of structure 3:4, thereby preventing transcription termination. The various structures have the following calculated free energies of formation¹⁹: 1:2, $\Delta G = -4$ kcal mol⁻¹ (see text for discussion); 2:3, $\Delta G = -19.6$ kcal mol⁻¹; 3:4, $\Delta G = -21.6$ kcal mol⁻¹. The hypothetical structure formed by pairing of nucleotides 3–19 with 23–27 has a theoretical $\Delta G = -1.8$ kcal mol⁻¹. To simplify discussion of potential secondary structures we have assumed that this structure forms. We know of no function for such a structure.

some stalling over a crucial segment of the transcript and not by the tryptophan deficiency *per se*.

If the role of the ribosome is simply to mask appropriate segments of the *trp* transcript, we could simulate such masking by deleting the corresponding segment of the transcript. Accordingly, we produced a set of overlapping deletions having both termini within the leader region. We selected those deletions that also removed the leader peptide ribosome-binding site and translation start codon to avoid the complication of translation. We describe here the *in vivo* analysis of the effects of these deletions on attenuation.

Analysis of deletion mutants lacking segments of the *trp* leader region of *S. marcescens*

Deletions around the *Hpa*I site, nucleotides 32–37, in the *trp* leader region were produced *in vitro* by exonuclease digestion, as described in the accompanying paper¹⁸. Each deletion mutant was characterized by DNA sequence analysis to determine the deletion junction. The leader regions containing deletions were then introduced into the chromosome in single copy form for operon expression studies. The recipient for these studies was an *E. coli* strain that lacked the *trp* repressor and had a deletion of *trpE* (*trpR* Δ *trpLE1413*). This deletion removes the *E. coli* *trp* attenuator and *trpE*, resulting in the production of sufficiently high levels of the *trpD* protein of *E. coli* to activate a broad range of levels of *S. marcescens* *trpE* protein. Cultures were either grown with excess tryptophan or starved of this amino acid. As the translation start codon of the leader transcript was deleted in all the deletions analysed, the +Trp values are most relevant to considerations of operon expression.

Figure 2a summarizes expression data for four deletions that remove the leader translation start codon. With the exception of deletion 221, operon expression, and presumably transcription readthrough, is reduced to 8–18% of the value obtained with the wild-type leader region. As documented in the accompanying paper¹⁸, deletions that prevent translation of the peptide-coding region cause increased termination, or superattenuation. Presumably, in such strains secondary structure 1A–1B:2A–2B forms and prevents pairing of 2A with 3. Thus, 3:4, the terminator, forms at high frequency. In this explanation it is assumed that when the coding region is translated in wild-type, structure 2A:3, the anti-terminator, forms occasion-

ally, leading to a moderate level of transcription readthrough. Deletion mutant 221 requires additional explanation. The sequence created by the deletion in this strain, CGGCC.AACAC (nucleotides 16–18 fused to nucleotides 56–58), is complementary to the 1A–1B junction (nucleotides 78–83, Fig. 2a) and conceivably could pair with this segment and thereby reduce the frequency of 1A–1B pairing with 2A–2B. Free 2A–2B would thus be available to generate the anti-terminator. Mutant 307, which differs from 221 by only one base pair (C is substituted by G at position 18), has fourfold lower expression. In agreement with this result, it can be calculated that this C→G substitution considerably reduces the theoretical estimate of the free energy of the potential secondary structure (from $\Delta G = -5.6$ to $\Delta G = +1.6$ kcal mol⁻¹).

The importance of segments 1A and 1B in the regulation of transcription termination is evident from analyses of deletions having endpoints in these segments (Fig. 2b). In general, as more of segment 1A–1B is removed, the level of observed *trpE* expression increases until it reaches values of ~400%, reflecting the absence of termination at the attenuator. Mutant 206 is an

Table 1 The effect of starvation for various amino acids on readthrough transcription in the *S. marcescens* *trp* operon

Growth conditions	% Of pulse-labelled RNA annealed to <i>S. marcescens</i> <i>trpE</i> DNA	Relative <i>trpE</i> mRNA levels
Non-starved	0.13	(100%)
Trp-starved	0.62	476
Arg-starved	0.53	410
His-starved	0.46	353
Pro-starved	0.15	115

The numbers in the last column indicate the average structural gene *trpE* mRNA level expressed by λ Sm4 derivatives in *E. coli* starved for the particular amino acid, relative to the value obtained with a non-starved culture (100%). The results are averaged from six experiments. The error of the measurement is 20% of the number given. The non-starved λ Sm4 lysogen (*E. coli* background: LS993 *trpR* Δ *trp* *ED24* *argE* *his* *pro* *ilo*) was grown in minimal medium containing 0.05% casein hydrolysate, 0.2% glucose and 50 μ g ml⁻¹ of required amino acids. Samples of this culture were filtered, washed and starved of individual amino acids for 5 min before being pulse-labelled for 30 s at 30 °C with ³H-uridine. The labelled RNA was hybridized in duplicate to plasmid pITS9, which carries *S. marcescens* *trpE*, and the vector plasmid pACYC184 (ref. 24). The difference between these two values is indicated in the second column as a net per cent of the added label which was bound to filters. It is taken as a measure of readthrough transcription beyond the attenuator.

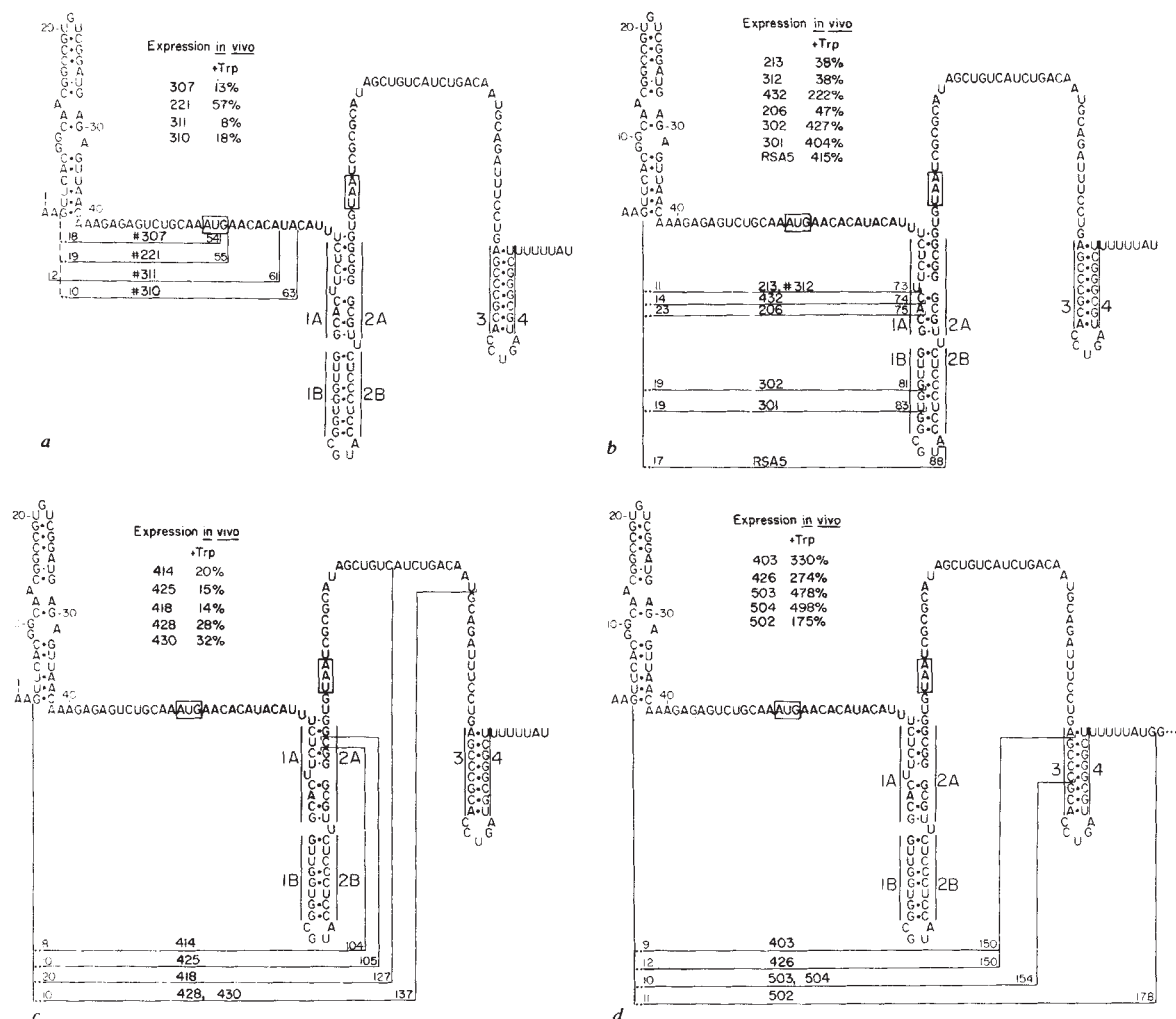


Fig. 2 Deletions in the *S. marcescens trp* leader region that remove the putative translation start codon and regions beyond, increase or decrease transcription termination at the attenuator. Small numbers at the end of the deletion bars indicate the outermost positions of the deleted nucleotides. Deletions are grouped according to the position of the right-hand endpoints. Expression *in vivo* represents the level of *trpE*-encoded anthranilate synthetase produced in *E. coli* strains carrying a single copy of the mutant *S. marcescens trp* leader region followed by an intact *trpE* gene. The numbers correspond to the per cent of the wild-type (*S. marcescens*) *trpE* expression in the same strain (W3110 *trpR* Δ *trpLE1413 inaA2*/λgtSm4 wild type) grown with an excess of tryptophan. The results are averaged from at least three separate experiments. The experimental error is 20% of the values shown. For the determination of *trpE* enzyme levels the strains were grown at 30 °C in minimal medium²⁵ supplemented with 0.1% glucose, 0.05% acid-hydrolysed casein and 50 μg ml⁻¹ of L-tryptophan. Cells were collected at a density of 6 × 10⁸–7 × 10⁸ per ml, washed with saline, resuspended in 0.1 M Tris-HCl (pH 7.8) and disrupted by sonication. Cell debris was removed by centrifugation and the levels of anthranilate synthetase component I were determined in the ammonia assay²⁶. DNA sequence analyses were carried out by the method of Maxam and Gilbert²⁷. *a*, Deletions which end before segment 1 cause superattenuation (see accompanying paper)¹⁸. *b*, Deletions which end in or near segment 1B generally cause high readthrough. *c*, Deletions which end between segments 2 and 3 cause increased termination. *d*, Deletions which remove all of segment 2 and part or all of segments 3 and 4 cause high readthrough.

exception to this pattern. Note, however, that the deletion in 206 allows nucleotides 17–20 (CCGU) to pair with segment 2A at positions 104–107 (GCCG), thereby conceivably restoring hydrogen bonding between segments 1 and 2.

The free energy of formation for the wild-type base-paired 1:2 structure, calculated by currently recommended rules¹⁹, is $\Delta G = -4$ kcal mol⁻¹. We believe that this value may be a gross underestimate of the true stability of this structure. We feel that the theoretical bases for stability predictions do not take into account the unusual sequences present in structure 1:2. In particular, the high pyrimidine content of the interior loops and the possible contribution of tertiary interactions may markedly influence stability. From our observations we tentatively conclude that structure 1:2 is moderately stable. An alternative hypothetical structure can be deduced for 1:2, and will be discussed below.

Deletions which remove segments 1 and 2 result in superattenuation—*trpE* expression four- to sevenfold lower than that in wild-type (Fig. 2c). The behaviour of this class of deletions establishes that the terminator, 3:4, is the only secondary structure signal necessary to cause transcription termination at the attenuator. Deletion of increasing portions of the loop

region separating segments 2 and 3 confirm this conclusion; the extent of termination in mutants of this class is essentially the same as in deletion mutants that simply remove the translation start codon. Thus, superattenuation is observed whether or not 1:2 exists and can form.

The final set of deletions are those having endpoints in region 3 or beyond 4 (Fig. 2d). These result in high levels of operon expression, suggesting that there is little or no termination. Surprisingly, deletions 403 and 426 largely eliminate termination yet they disrupt only one base pair of structure 3:4 (A–U, nucleotides 150 and 170). These deletions change the calculated ΔG for 3:4 from -21.6 kcal mol⁻¹ to -19.4 kcal mol⁻¹. We do not know why these mutations have such a profound effect. In *E. coli*, the predicted stability of structure 3:4 in termination-deficient mutants that show two- to fourfold increased expression of the operon *in vivo* is reduced by ~50% (refs 5, 20). All these mutations, unlike the *S. marcescens* deletions, affect the central GC pairs of the terminator. Deletion 502 removes the entire terminator but the observed expression in this mutant is not as high as in deletion 503. Studies *in vitro* indicate that there is no transcription termination on templates with this deletion (I.S. and C.Y., unpublished). We hypothesize

that as this deletion ends 26 nucleotides upstream from the *trpE* structural gene, it interferes with ribosome binding and initiation of translation of *trpE* mRNA.

A curious effect of tryptophan starvation

All the deletions described here remove the start codon for the leader peptide and consequently eliminate translation of the peptide-coding region. Nevertheless, we have found that every deletion mutant in which there is transcription termination at the attenuator responds to tryptophan starvation by exhibiting a 1.5–2-fold increase in anthranilate synthetase levels (data not shown). In contrast, deletion mutants that do not terminate transcription at the attenuator have identical anthranilate synthetase levels whether starved of tryptophan or not. The explanation for this observation is unknown. Perhaps the small increase is due to some general effect of starvation on transcription termination; this effect is being investigated further.

Alternative RNA secondary structures in the regulation of attenuation

We have examined the effects on gene expression of 21 deletions with endpoints entirely within the leader region of the *trp* operon of *S. marcescens*. Although our functional analyses were carried out in *E. coli*, we have shown that in this heterologous system the wild-type *trp* operon of *S. marcescens* is regulated by attenuation in a qualitatively identical manner to the *trp* operon of *E. coli*. We find that deletions within the *S. marcescens* leader region which remove the leader peptide start codon and different segments of the region beyond may increase, decrease or have no effect on transcription termination at the attenuator. The positions of the deletion endpoints and their effect on gene expression are summarized in Fig. 3. It is apparent that as the right-hand endpoint extends further into the leader region, transcription termination increases, then decreases, then increases again and finally is eliminated entirely compared with the wild-type. These findings are most readily explained in

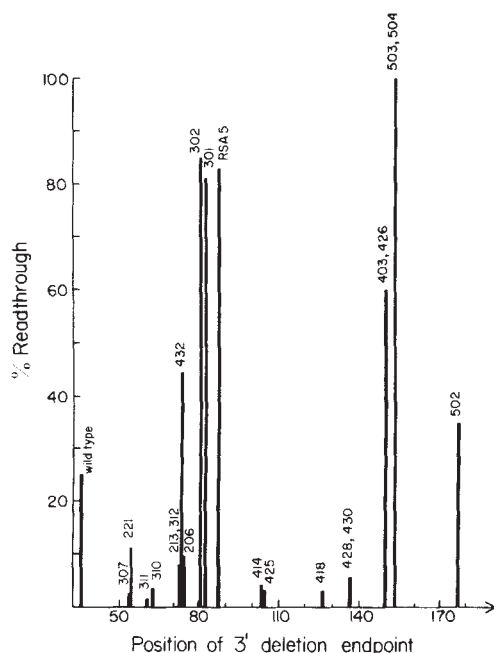


Fig. 3 Positions of right-hand deletion endpoints in *S. marcescens* *trp* leader mutants and the effect on *trpE* expression. The height of each bar represents the level of anthranilate synthetase produced during growth with tryptophan relative to the level observed in deletion mutants 503 and 504 (set at 100). The range of *S. marcescens* *trp* operon expression varies 30–60-fold in different deletion mutants. Maximum transcription termination is observed in the translation-defective deletion mutant, 311, in which readthrough occurs at ~10% that in wild type. By comparison, in the *E. coli* initiation codon point mutant, *trpL29*, readthrough is ~25% that in wild type²⁰.

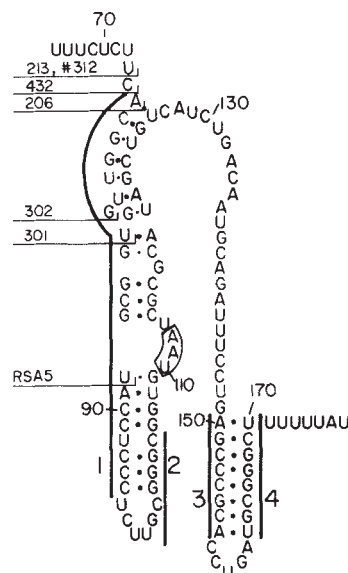


Fig. 4 An alternative potential secondary structure in the *S. marcescens* *trp* leader transcript. The secondary structure labelled 1:2 is formed by the pairing of segments of the transcript other than those paired in the structures depicted in Figs 1 and 2. Note that segment 2 is identical in all the structures and can pair with either segment 1 or segment 3. The endpoints of the deletions described in Fig. 2b are indicated. Some of these deletions reduce termination at the attenuator (see text). The calculated ΔG value for the hypothetical structure shown involving nucleotides 75–126 is $-23 \text{ kcal mol}^{-1}$; and for the portion of the structure involving nucleotides 88–109, $-13 \text{ kcal mol}^{-1}$. In making these estimates it was assumed that a G · U base pair is equivalent to an A · U base pair.

terms of the alternative secondary structure models schematically represented in Fig. 1.

Another potential 1:2 RNA secondary structure exists (Fig. 4); this structure is predicted to be more stable than structure 1:2 as drawn in Figs 1 and 2. However, the effect of deletions 432, 302 and 301 is inconsistent with the structure in Fig. 4, for these deletions should not reduce termination if structure 1:2 in Fig. 4 is correct, but should relieve termination according to structure 1:2 in Figs 1 and 2. In view of our findings, we prefer the secondary structure presented in Figs 1 and 2.

Several studies on attenuation in the *trp*^{5-7,20,21} and *his*^{22,23} operons support the assumption that changes in the secondary structure of the leader transcript mediate the regulatory response. The experiments described here were designed to test this assumption. The results obtained are in agreement with the secondary structure model and the expected consequences of deleting specific RNA segments that can participate in base pairing. Thus, we believe that the ribosome translating the leader transcript has no role other than sterically to prevent base pairing by the segment of RNA with which it is in contact. This conclusion is supported by the results of *in vitro* transcription studies with restriction fragments bearing the *Serratia* deletions described here (I.S. and C.Y., in preparation). In these studies we have found that in a minimal transcription system consisting of restriction fragments and RNA polymerase, the same pattern of increased termination and termination relief seen *in vivo* with our deletion mutants is reproduced *in vitro*. We conclude, therefore, that when the RNA terminator forms, the transcribing RNA polymerase molecule recognizes that structure and transcription termination occurs. The role of translation, ribosome stalling and alternative RNA secondary structure formation, is simply to regulate formation of the terminator.

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Superattenuation in the tryptophan operon of *Serratia marcescens*

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Deletions were generated in vitro in the leader region of the tryptophan operon of Serratia marcescens and subsequently incorporated into the Escherichia coli chromosome in single copy form. Deletions which removed the translation start codon for the leader peptide or which ended in the ribosome recognition region preceding the leader peptide coding segment, caused superattenuation, that is, increased transcription termination at the attenuator. Apparently, the capacity to initiate translation of this coding region modulates expression of the operon.

THE tryptophan (*trp*) operon of each of the six enterobacterial species that have been studied (refs 1–5 and M. Blumenberg and C. Y., in preparation) is controlled at the level of transcription termination by a mechanism called attenuation. In each *trp* operon, translation of a short peptide coding region in the initial, leader segment of the transcript seems to regulate the formation of alternative secondary structures, one of which is recognized by RNA polymerase as a transcription termination signal. It has been proposed that in cells which have an adequate supply of Trp-tRNA^{Trp}, translation proceeds to the end of the coding region, thereby favouring formation of the RNA secondary structure that serves as the termination signal. However, when most of the cellular tRNA^{Trp} is uncharged, the ribosome translating the peptide coding region of the transcript stalls at one of two adjacent Trp codons, generating an alternative RNA secondary structure that excludes formation of the termination structure.

Studies with *Escherichia coli* mutants defective in the regulation of transcription termination at the *trp* operon attenuator⁶ suggest that translation of the peptide coding region is utilized for two purposes. First, translation mediates the regulatory response to changes in the level of charged tRNA^{Trp}. Second, translation establishes the moderate level of transcription read-through that is characteristic of cultures growing with excess tryptophan. Thus, there is fourfold increased transcription termination at the attenuator of a leader mutant in which the start codon for the leader peptide has presumably been inactivated. This observation suggests that attenuation is modulated by the ability to initiate translation as well as by tryptophan availability.

Here we describe the production of deletions within the *trp* leader region of *Serratia marcescens* and analyse the effects of those that alter RNA segments believed to be essential for

initiation of synthesis of the leader peptide. Our findings indicate that elimination of translation initiation results in increased transcription termination at the attenuator, or superattenuation.

Directed *in vitro* deletion mutagenesis in the *trp* leader region of *S. marcescens*

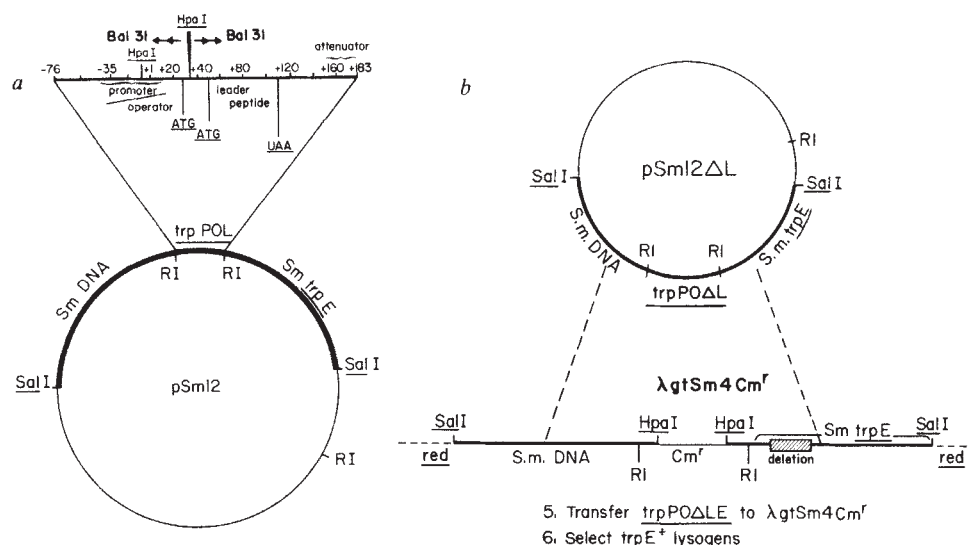
Deletions were generated around the *Hpa*I site at position 34/35 of the leader region by limited digestion with *Bal*31 exonuclease⁷ (Fig. 1a). This exonuclease acts quasi-processively at both 3' and 5' termini of double-stranded DNA to release 5' mononucleotides; digestion predominantly produces blunt ends⁷. There are two *Hpa*I sites in the *trp* regulatory region; *trp* repressor was added to protect the *Hpa*I site in the promoter. However, as *Bal*31 digestion of the promoter would inactivate it, strains with such deletion plasmids would not be detected in the subsequent step requiring *trpE* expression. Following *Bal*31 digestion, ligation and transformation, plasmids were isolated from individual transformants and screened for deletions in the desired region by examining the size of the single *Eco*RI fragment that contains the *trp* regulatory region, and by mapping restriction sites within the fragment. The distribution of deletion endpoints in the mutants was further characterized by DNA sequence analysis⁸ and is shown in Fig. 2. There is asymmetry in the length of the segment deleted on either side of the *Hpa*I site, presumably because deletions extending into the *trp* promoter are not recovered.

Expression of deletion mutants

The effect of the deletions on attenuation was determined after introducing each deletion into the chromosome, in single copy form, as outlined in Fig. 1b. Briefly, phage λ gtSm4Cm⁺ was grown in a bacterium with each deletion plasmid and the lysate obtained was used to transduce a *trpR trpE* deletion strain to Trp⁺. Control experiments showed that lysogens prepared with a homologous phage that has *trpE*⁺ and the 22 base pairs

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Fig. 1 Deletion mutagenesis of the *trp* leader region of *S. marcescens* (a) and introduction of deletions into the *E. coli* chromosome (b). Deletions were generated in plasmid pSM12⁺. The *EcoRI* fragment containing the regulatory region of the *S. marcescens* *trp* operon is shown enlarged at the top of the figure (a). Number +1 corresponds to the presumed site of transcription initiation. The 'in-phase' AUG and UAA codons indicate the potential coding region for the leader peptide. Deletions were generated around the *HpaI* site at position 34/35. To ensure preferential cutting at this site, partial digestion with *HpaI* was done in the presence of *E. coli* *trp* repressor, which is known to protect the *HpaI* site at position -11/-12 from cleavage⁴. 20 µg of partially digested DNA was treated with *Bal31* exonuclease in a reaction mixture containing 20 mM Tris-HCl pH 8.1, 200 mM NaCl, 12 mM CaCl₂, 12 mM MgSO₄ and 1 mM Na₂EDTA in a 300 µl total volume. The reaction mixture was incubated on ice and samples were taken after 1 and 15 min, the mixture was transferred to room temperature, and samples were taken after an additional 1, 4 and 6 min. The reactions were stopped by adding 10 mM EDTA pH 8 and the DNA precipitated with ethanol. 1 µg of DNA from each sample was resuspended in 200 µl of ligation buffer (60 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP). The mixtures were incubated in the presence of T4 DNA ligase for 2-4 h at room temperature and used directly to transform *E. coli* strain JA221 (C600 *r⁻m⁺ Δ trpE5 thr leu recA*) to Trp⁺. The plasmid DNA from the transformants was analysed by digestion with *EcoRI* and *HinfI* restriction enzymes. Approximately 25% of the plasmids carried a deletion of the *HinfI* site at position 43/47 and preserved the integrity of the *EcoRI* sites bordering the *trpPOL* region. These plasmids were used to transform W3110 *trpR Δ trpE5 trpA2* to Trp⁺ and were analysed further by sequencing⁸. The regulatory region containing each deletion was transferred to phage λ and then into the *E. coli* chromosome by general recombination and lysogenization as shown schematically in b. To promote recombination, *E. coli* *recA⁺* strains containing the mutant plasmids were infected lytically with bacteriophage λgtSm4Cm^r. The lysates were used to lysogenize strain W3110 *trpR Δ trpE5 trpA2* at a permissive temperature (30 °C). The recombinants were identified as Trp⁺ prototrophs, temperature sensitive and unable to grow in the presence of 25 µg ml⁻¹ of chloramphenicol. To ensure single copy lysogeny the infections were performed at low multiplicity (<0.001). Bacteriophage λgtSm4Cm^r was constructed by recombinant DNA techniques using DNA from λgt4 (Ron Davis, personal communication), pSM12⁺ and pACYC184¹⁷.



1. Cut pSM12 with *HpaI* endo
2. Treat with *Bal31* exo
3. Ligate to circularize
4. Select *trpE⁺* transformants

5. Transfer *trpPOLΔLE* to λgtSm4Cm^r
6. Select *trpE⁺* lysogens

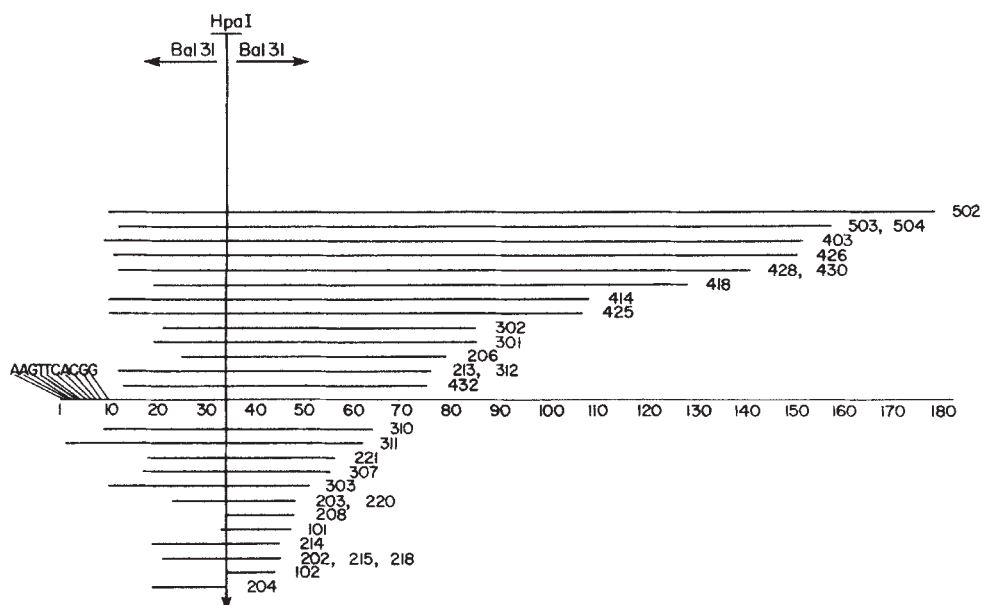
preceding it intact, but lacks the *S. marcescens* *trpPOL EcoRI* fragment, have less than 0.1% of the wild-type level of anthranilate synthetase (*trpE⁺*) activity and are phenotypically Trp⁻. Thus, *trpE⁺* expression from our leader deletion lysogens must originate at the *trp* promoter in the *EcoRI* segment. This conclusion was substantiated by introducing *trpR⁺* into some of these strains and showing that *trpE* expression was under *trp* repressor control.

In Fig. 3 we indicate the sequence of the leader transcript segment containing each deletion junction. Also shown is *trpE* expression relative to that of a control culture containing the unaltered wild-type leader region. The first four deletion mutants, 204-208, have the wild-type sequence in the ribosome binding region for leader peptide synthesis⁹. These strains

exhibit wild-type levels of anthranilate synthetase and respond to tryptophan starvation by a four- to five-fold increase in synthetase levels. The fifth deletion mutant, 102, has half the wild-type levels both in the presence of and following starvation for, tryptophan. Note that the sequence in the ribosome binding region is altered although homologous GAG sequences are located slightly earlier than in wild type. We interpret the findings with this mutant as indicating that translation initiation does occur at the 102 AUG start codon but is somewhat reduced in frequency relative to that of the wild type.

The second group of deletions, represented by seven independently isolated deletion plasmids, have a considerably different effect. Strains with these deletions have 8-10-fold reduced anthranilate synthetase levels compared with the wild type;

Fig. 2 Distribution of deletion endpoints in the *S. marcescens* *trp* operon leader region. Numbering is from the presumed site of transcription initiation. The site of *HpaI* cleavage and direction of *Bal31* digestion are indicated by arrows. The nucleotide sequence of the first 10 residues is shown on the left.



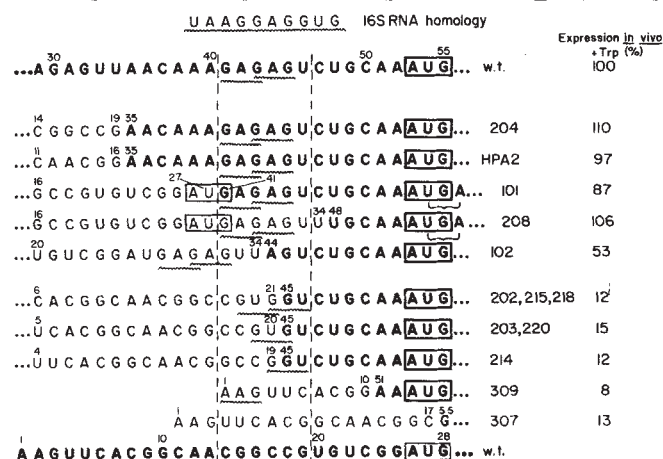


Fig. 3 The nucleotide sequence of the segments of the leader transcript containing deletion junctions. The former positions of the fused nucleotides are indicated by small numbers. The mutant sequences are aligned at wild-type position 55 and compared with the wild-type sequence at the top and bottom of the figure. The presumed initiation codon is boxed by a heavy line, while the AUG which is thought not to function as a translation initiation codon is boxed by a thin line. Nonsense UGA codons which are in phase with AUG codons are bracketed. Complementarity to 16S rRNA is underlined by wavy lines. Vertical broken lines show the region thought to be important in ribosome binding and initiation of leader peptide synthesis. Numbers referring to expression *in vivo* refer to per cent of wild-type *trpE* expression measured in the same strain containing the wild-type region. Growth conditions and assays of *trpE* enzyme activity are described in Fig. 2 legend of the accompanying paper.

these levels are increased only slightly when the strains are starved of tryptophan (see accompanying paper¹⁰). These deletions have in common changes in the nucleotide sequence of the ribosome binding region. However, in each case we can indicate some new sequence that is complementary to the 3' end of 16S rRNA. We interpret these findings as indicating that in these deletions there is little or no translation initiation at the normal AUG start codon, and that the absence of translation is responsible for reduced *trpE* expression. The final mutant, 307, deletes through the start codon; it behaves indistinguishably from the preceding group of seven.

At the bottom of the Fig. 3 we present the sequence of the first 28 nucleotides of the transcript. There is a potential AUG start codon at positions 26–28 that is in-phase with the AUG at 53–55. We believe that this AUG codon is not used as a site of translation initiation, for the following reasons. First, deletions which remove this region are phenotypically indistinguishable from wild type. Second, fusion of this region in deletions 101 and 208 places the first AUG out of phase with the second AUG, but in-phase with a UGA stop codon that overlaps that second AUG. If the first AUG were used as a site of translation initiation we would expect that translation from this site would

interfere with in-phase translation of the tryptophan codons, which must occur in view of the tryptophan starvation response. Thirdly, if translation were initiated in wild type at the first AUG, several amino acid residues including Arg would be added at the amino-terminal end of the leader peptide. Yet arginine starvation relieves transcription termination at the attenuator¹⁰, implying that in the absence of this amino acid a ribosome can reach—and stall at—the Arg codon located immediately following the tandem Trp codons. Finally, there are no sequences resembling a Shine–Dalgarno sequence in the appropriate segment of the transcript (Fig. 3).

Significance of reduced operon expression in mutants with altered leader peptide translation initiation regions

The increase in transcription termination at the attenuator in deletion mutants implies that in wild type there is some mechanism responsible for establishing a moderate level of transcription readthrough and hence operon expression in cultures growing with excess tryptophan. The superattenuating deletions have in common changes in the sequence of nucleotides believed to be required for efficient ribosome binding; these changes presumably result in reduced initiation of synthesis of the leader peptide.

Alternatively, the leader segments could contribute to an RNA secondary structure involved in regulation. However, as deletions 204, HPA2, 101 and 208 lack segments of the same region yet exhibit wild-type expression and response, it seems likely that a defect in translation initiation is responsible for the observed reduction of expression. This interpretation leads to the conclusion that normal translation of the leader peptide coding region proceeds concomitantly with transcription and that ribosome movement or position is responsible for the moderate level of expression seen in wild type. In accordance with the model proposed previously² and supported by the findings in the accompanying paper¹⁰, we conjecture that the anti-termination secondary structure, 2:3 forms occasionally when a ribosome translates the peptide coding region. This could occur by transitory masking of segment 1 by a sluggish ribosome, occasional pairing of segments 2 and 3 as the ribosome dissociates from the stop codon, or masking of segment 1 by a second ribosome on the transcript. The role of transcriptional pausing¹¹ in the synchronization of transcription with translation must be investigated before these or other explanations can be realistically distinguished.

Regardless of the basis for the moderate expression in wild type, our findings indicate that superattenuation could be used by the bacterium to achieve a level of *trp* operon expression well below that characteristic of cultures growing in the presence of excess tryptophan. Thus, if a cell were incapable of initiating

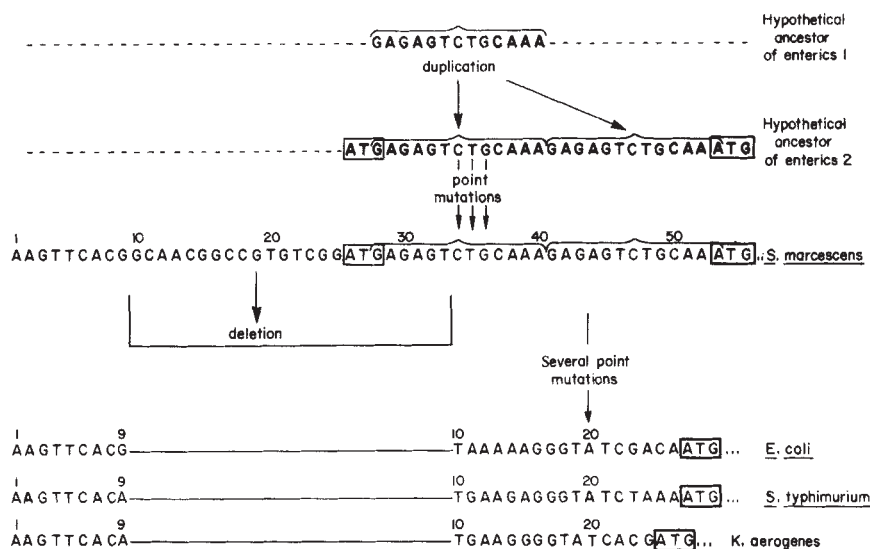


Fig. 4 A sequence of possible evolutionary events leading to the present-day nucleotide sequences of the *trp* leader regions of various enterics. In reality the postulated deletion event would have taken place in a common ancestor of the Enterobacteria, which could have had a nucleotide sequence somewhat similar to that of *S. marcescens*.

translation of the leader coding region, for whatever reason—a deficiency of formylating activity, lack of an initiation factor or a drastic decrease in the number of available ribosomes—this would be recognized as a signal to superattenuate. If this feature is common to all instances of attenuation control of amino acid biosynthetic operons, it is apparent that attenuation may be used generally to attain a greater range of operon expression than had previously been thought possible. Two mutations of the *his* operon leader region support this hypothesis^{12,13}. They are phenotypically similar to our superattenuating deletions and are altered in the region potentially involved in translation initiation in *his* leader peptide synthesis. Studies on *trp* mRNA synthesis in *trpR* strains of *E. coli* following a nutritional or carbon source shift-up suggest that superattenuation may occur naturally in the wild-type operon¹⁴.

Tryptophan starvation in strains with either the *E. coli* or *S. marcescens* wild-type *trp* operon results in operon expression approaching the level seen in leader deletion mutants that lack the attenuator. This finding suggests that in tryptophan starvation conditions translation initiation must be so efficient that virtually every *trp* mRNA molecule has a ribosome stalled over the Trp codons of the leader transcript. The view that the leader ribosome binding site is efficient is supported by *in vivo* and *in vitro* studies^{15,16}.

Possible duplication in the *S. marcescens* leader region

Comparison of the initial segment of the *trp* leader regions of several enterobacteria (Fig. 4) reveals that *S. marcescens* seems to have a 25-base pair insert. This region in *S. marcescens* contains a putative start codon that is in phase with the functional start codon at 53–55 (Fig. 2). The role, if any, of this

extra segment is brought into question by the findings discussed earlier that deletions that remove this segment have no detectable effect *in vivo* on attenuation and its regulation. The RNA segment bases 3–19 can form a weak secondary structure with segment base 23–37 (see accompanying paper¹⁰); however, we know of no functional role for this pairing. We must consider the possibility, therefore, that the insert is a remnant of an ancestral sequence that once had a function. The sequence of a segment of the insert is strikingly similar to a proximal sequence, suggesting that the insert arose as a consequence of duplication. Presumably, a portion of one of the duplicated segments was deleted in the evolution of leader regions of the *E. coli* type. A sequence of possible evolutionary events is depicted in Fig. 4. One might imagine that the duplication arose initially in response to selection for a more efficient ribosome binding site or a requirement for simultaneous translation by two ribosomes. Some time thereafter the weaker of the two ribosome binding sites was inactivated to give the present-day sequence of *S. marcescens*, or deleted to produce the *E. coli* arrangement. In this regard it is of interest that *trpG* and *trpD* are separate, adjacent genes in *S. marcescens*⁴ whereas in *E. coli* and other enterobacteria these genes are fused in what seems to be a more advantageous arrangement. Both this instance and the existence of the apparent non-functional insert in the *S. marcescens* *trp* leader region suggest that the *trp* operon of *S. marcescens* may be more primitive than its counterpart in *E. coli*. Support for this view awaits specific tests of the functional significance of the different genetic arrangements.

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LETTERS TO NATURE

Optical identification of X-ray source H0139–68 with an AM Herculis-type system

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We report here the identification of the soft X-ray source H0139–68 with a star at RA = 01 h 39 min 37.5 s, dec = –68° 08' 32" (1950). Time-resolved spectrophotometry of the star over 3 h shows a V magnitude variation between 14.9 and 16.4 with a period of ~110 min. The spectra are dominated by emission lines of neutral hydrogen, neutral helium and singly ionized helium throughout the period. The line ratios of the Balmer lines H_γ/H_β and the He I lines are flatter relative to case B recombination values, while the He II ratios are consistent with optically thin recombination. The mean radial velocity of the emission lines varies with the photometric period roughly sinusoidally from 340 to –150 km s^{–1}. The continuum is very red at maximum light, flat at minimum and shows strong polarization. These optical characteristics of the candidate star are similar to those of the magnetic cataclysmic variable AM Herculis.

The candidate star is marked X in Fig. 1, along with the X-ray source error circle of radius 36 arcs (ref. 1). Spectrophotometric observations of the candidate star were obtained on the night of 6 October 1981, with the 1DPCA² at the f/8 cassegrain focus of the Mount Stromlo Observatory 1.9-m telescope. We obtained 17 spectra of the candidate star in the time interval 15 h 20 min UT to 18 h 20 min UT, with a

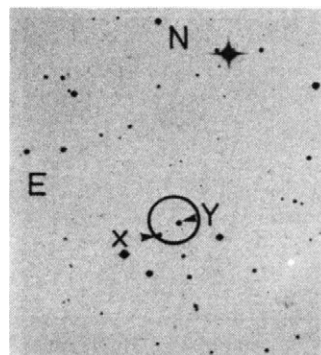


Fig. 1 The circle of diameter 72 arcs defines the X-ray source location, and X marks the optical identification with H0139–68. The coordinates of X are RA = 01 h 39 min 37.5 s, dec = –68° 08' 32" (1950). The star marked Y shows a late-type spectrum with no variability.

Note that we do not see any stellar absorption features in the spectra at any phase. We deduce that any underlying stellar continuum contribution is $<10\%$ of the measured flux implying an apparent V_{mag} of 19 or fainter for any unseen stellar system.

More information on the line-emitting region can be obtained from the radial velocity data. The stronger H I, He I and He II lines have been fitted with gaussians to determine the line centres and line widths for each line of each scan separately. The measured line widths (σ of the fitted gaussian, expressed as velocity dispersion) have been corrected for instrumental broadening by quadratically subtracting the measured arc line width of 130 km s^{-1} . We are unable to detect any multiple components in the lines at any phase, but the instrumental resolution is insufficient to detect the presence of sharp components as seen in AM Herculis⁸. The radial velocity curve given in Fig. 4a shows a roughly sinusoidal variation with mean amplitude of $2K = 490 \text{ km s}^{-1}$, asymmetrically distributed about velocity zero (with respect to the Sun). The line widths of the emission lines (Fig. 4b) also change with phase, with both maximum recessional velocity and maximum line width occurring near maximum light. The higher ionization species He I, He II display higher maximum recessional velocity and higher broadening at maximum than do the H I lines. These differences are significantly greater than the mean errors for single velocity or width determinations, which are indicated in Fig. 4b.

Part of the asymmetry in the radial velocity curve may be due to the unknown systemic velocity, but the large amplitude and variation in the line width with phase imply the presence of a gas stream. The radial velocity of the system is then dominated by streaming motions of a single mass transfer stream directed towards the primary. The orbital motion component for a typical system (with a red dwarf of mass $\sim 0.2 M_{\odot}$ and a white dwarf of mass $0.3 M_{\odot}$) is derived as 80 km s^{-1} , with a maximum of 170 km s^{-1} for a white dwarf mass of $\sim 1.4 M_{\odot}$. Higher orbital velocities imply the presence of a large mass secondary which would be too luminous not to be seen. This

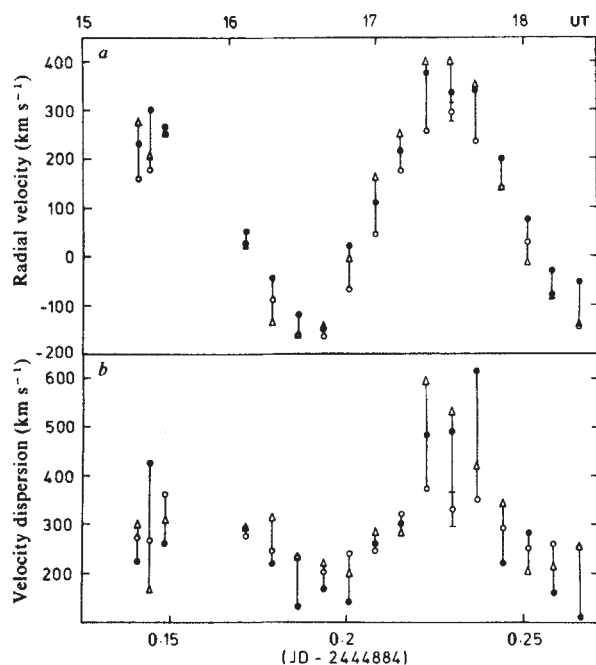


Fig. 4 *a*, The emission line radial velocities (w.r.t. Sun) of H I (○), He I (●) and He II (△) as a function of time of observation: 2σ error bars for a single observation are shown. *b*, The velocity dispersion (σ of fitted gaussian) of the emission lines as a function of time of observation. The instrumental broadening measured on arc lines has been quadratically subtracted. This figure shows that both maximum radial velocity and maximum velocity dispersion are higher for higher ionization species. There is some indication of phase shift between the different species.

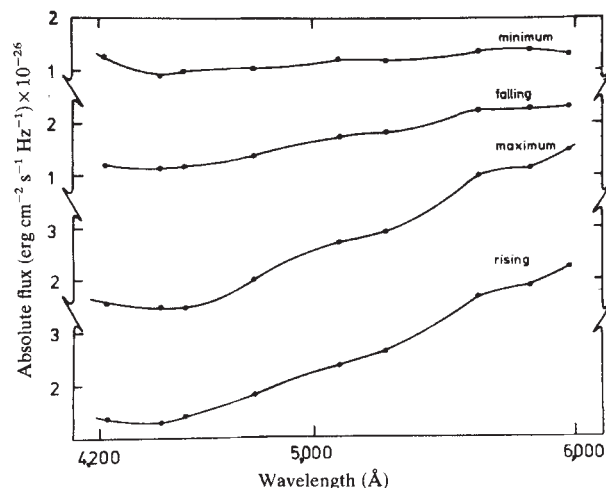


Fig. 5 The continuum flux as a function of phase. The marked points are the mean fluxes in regions away from emission lines, with freehand fits to the points shown. There is indication of cyclotron absorption troughs at maximum, at $\lambda 4,500$ and $\lambda 5,400$.

mass transfer stream is consistent with models of AM Herculis^{9,10} and VV Puppis¹¹ in which a single mass transfer stream from a late-type star overflowing its Roche Lobe, is funnelled down onto a magnetic white dwarf primary, with the magnetic field lines constraining the mass flow. In this model the velocity width would be due to the line-of-sight sampling of a range of velocities along the falling gas stream. The varying angle between the line-of-sight and the gas stream would account for the variation in line width with phase. The higher maximum recessional velocity and larger width of the higher ionization species, indicates that they originate closer to the primary, where the velocities are higher.

The absolute energy distribution in the continuum between 4,200 and 6,000 Å has been computed by averaging the line-free regions of each spectral scan, and plotting these points against wavelength for four different parts of the light curve (Fig. 5). It can be seen that the continuum is reddest during the rising and bright phases of the system, while it is flat during the faint and falling phases. This variation of the continuum is also seen in AM Herculis¹⁰ and VV Puppis¹¹.

If we accept the model of matter transfer onto a magnetic white dwarf primary, then we might expect to see both linear and circular polarization in the optical continuum, due to the presence of cyclotron radiation at optical frequencies. Note that there is a suggestion of cyclotron absorption harmonics at 4,500 and 5,400 Å in the continuum at maximum (Fig. 5) and preliminary linear polarization observations indicate that the continuum is polarized as high as 7% close to maximum¹².

In summing up the evidence for this optical identification, we note that periodic variation of the continuum, line emission, and radial velocity of the candidate star reveals that the star is an eclipsing system. The spectra have bright emission lines of H I and He I originating in optically thick and high density regions in the system. The nearly sinusoidal radial velocity and velocity width variations with phase indicate the presence of a gas stream falling into the primary. The continuum colour varies with phase. Linear polarization of the continuum supports the cyclotron origin for the continuum observed, implying the presence of a magnetic white dwarf in the system¹². Fast photoelectric photometry of the Star X indicates flickerings on a time scale of 100 s or less⁴. All these observed characteristics of the candidate star are seen in the binary system AM Herculis¹⁰ which has been identified with a soft X-ray source¹³. Within 36 arcs of the Star X there is the X-ray source H0139-68 (ref. 1), a source originally detected with the low energy detectors of HEAO A-2 experiment and found to be highly variable over periods as short as 3 h. No flux was detected above 0.4 keV indicating a soft spectrum. Observations with the image proportional counter of the Einstein observatory confirmed the

variability of the source and the presence of flickering on a time scale of 20 s or less.

Thus, the remarkable similarity between Star X and AM Herculis and the presence of the variable X-ray source H0139–68 very near to Star X, establish the Star X, a new AM Herculis type system, as the optical counterpart of H0139–68.

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Zonal harmonic model of Saturn's magnetic field from Voyager 1 and 2 observations

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An axisymmetric octupole model of Saturn's planetary magnetic field is proposed here. This three parameter model, characterized by the Schmidt-normalized spherical harmonic coefficients $g_1^0 = 21,535$ nT, $g_2^0 = 1,642$ nT and $g_3^0 = 2,743$ nT, is extremely efficient in representing the main magnetic field of Saturn and reconciling the *in situ* magnetic field observations obtained by Pioneer 11 with those obtained by the Voyager 1 and 2 spacecraft. Saturn's unique magnetic field configuration is thus not that of a simple displaced dipole but rather appears to be the axisymmetric part of a complex dynamo field. This result is consistent with Stevenson's model of the interior of Saturn¹, in which the differential rotation of a metallic fluid shell above the active dynamo region attenuates all non-axisymmetric components of the dynamo field.

The first *in situ* observations of the magnetic field of Saturn were obtained by the Pioneer 11 spacecraft in September 1979^{2,3}. Charged particle and magnetic field data both revealed a near perfect alignment of Saturn's magnetic dipole and rotational axes. Subsequent spherical harmonic analyses of the Pioneer 11 vector helium magnetometer observations⁴ led to

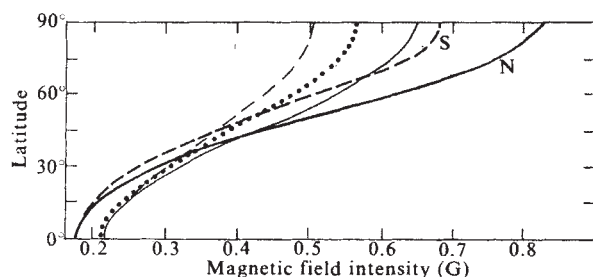


Fig. 1 Magnetic field intensity as a function of latitude on Saturn's oblate (flattening = 10.6) surface. Bold solid (dashed) line is the field intensity at north (south) latitudes corresponding to the Z_3 model (see text). Light lines indicate the field intensity of the offset ($\sim 0.04 R_s$) dipole model and the dotted line corresponds to a simple centred dipole with $0.21 G - R_s^3$ moment.

a model of Saturn's magnetic field ('Nominal 79 dipole') characterized by a dipole of $0.2 G - R_s^3$ (where 1 Saturn radius R_s is 60,000 km) aligned with Saturn's rotation axis and offset to the north along the rotation axis by $0.05 R_s$. [The units $G - R_s^3$ are dimensionally equivalent to standard units of magnetic dipole moment, the numerical value having been divided by the cube of the planetary radius.] Acuña *et al.*⁵ considered an axisymmetric potential expansion of Saturn's magnetic field to order $n = 2$ (quadrupole) and obtained from the Pioneer 11 fluxgate observations a g_2^0 coefficient of 1,500 nT, equivalent to a northward displacement of the dipole by $\sim 0.04 R_s$.

The Voyager 1 encounter with Saturn in November 1980⁶ presented a second opportunity to study Saturn's planetary magnetic field. Voyager 1 approached to within $3.07 R_s$ of Saturn and obtained the first observations of Saturn's magnetic field at high latitudes (to $41^\circ S$). Analyses of the Voyager 1 observations confirmed many aspects of Saturn's main field deduced from the Pioneer 11 observations but did not confirm the inferred northward displacement of the dipole^{6,7}. The near-equatorial Pioneer 11 approach to within $1.35 R_s$ yielded observations confined to a more limited range of latitude ($\pm 6^\circ$). The recent Voyager 2 encounter with Saturn (periapsis: $2.69 R_s$) in August 1981⁸ provided additional observations of Saturn's field at both high northern and southern latitudes ($\pm 30^\circ$). We now report the first internal field analyses to include the Voyager 2 vector magnetic field observations.

Our analysis of the magnetic field observations at Saturn uses the traditional spherical harmonic expansion of a scalar potential V from which the magnetic field is obtained through $B = -\nabla V$ (ref. 9). This representation requires that no sources be present within the minimum spherical shell enclosing the observations, and/or the region of representation, that is, that the volume of space $r_{\min} < r < r_{\max}$ contains no electrical currents ($\nabla \times B = 0$). The planetary field is characterized by the Schmidt-normalized coefficients g_n^m , h_n^m associated with the terms in V which decrease with increasing r . The external field is approximated by the first-order expansion corresponding to a uniform external field (G_1^0 , G_1^1 and H_1^1). The external field is due primarily to an equatorial azimuthal ring current of $\sim 7 \times 10^6$ A flowing between ~ 8 and $16 R_s$ (ref. 10). We therefore confine our analysis and modelling to observations obtained inside of $8 R_s$. We use 48-s averages of the vector magnetic field data

Table 1 Model fits of Saturn's magnetic field from Voyager 1 and 2 observations

	Unconstrained ($n_{\max} = 2$)			Axisymmetric ($n_{\max} = 3$)		
	V1	V2	V1+V2	V1	V2	V1+V2
g_1^0	21,302	21,912	21,439	21,576	21,433	21,535
g_1^1	455	23	-143	-	-	-
h_1^1	343	-176	143	-	-	-
g_2^0	535	1,908	1,882	1,715	1,643	1,642
g_2^1	318	-1,452	-515	-	-	-
g_2^2	1,016	1	500	-	-	-
h_2^1	-815	-551	-433	-	-	-
h_2^2	851	351	-36	-	-	-
g_3^0	-	-	-	2,686	2,583	2,743
G_1^0	-9	-10	-9	-11	-8	-10
G_1^1	0	-2	-1	0	-1	-1
H_1^1	2	-1	0	0	-2	0
r.m.s.	1.9	2.4	3.1	3.0	2.5	3.2

Equivalent offsets (R_s):

Δx	+0.0093	-0.0384	-0.0137
Δy	-0.0222	-0.0142	-0.0120
Δz	+0.0125	+0.0436	+0.0439

All entries are in nT.

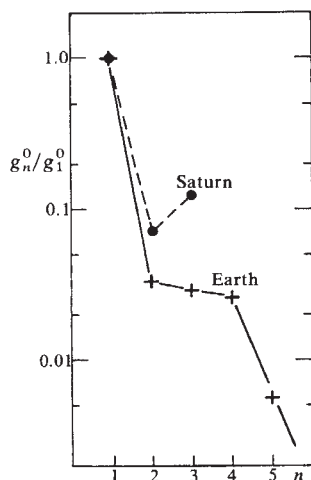


Fig. 2 Relative magnitude of the zonal harmonic (g_n^0) terms of the Earth's magnetic field and the Z_3 zonal harmonic model of Saturn's magnetic field.

obtained at 60-ms intervals to obtain least-squares model fits to the observations, with one exception. Data obtained by Voyager 2 during two brief roll manoeuvres performed near closest approach are included as field magnitude observations only, as the orientation of the spacecraft during these manoeuvres is uncertain ($\sim 2^\circ$). All of the models were obtained assuming equal weights for the individual observations and using the nonlinear inversion techniques described previously¹¹.

Our search for an appropriate physical model for Saturn's internal magnetic field can be traced through the model fits tabulated in Table 1. The notation $IjEk$ denotes a spherical harmonic expansion with internal (I) coefficients to degree and order j and external (E) coefficients to degree and order k . Analyses of the Voyager 1 and 2 observations individually in terms of a physical model including dipole ($n=1$) and quadrupole ($n=2$) terms only leads to best fit parameters which, with the exception of the main dipole g_1^0 term, show little reproducibility from one spacecraft encounter to another. In particular, the expected north polar offset (g_2^0) obtained from the Pioneer 11 observations^{3,4} is not to be found in the Voyager 1 (V1) $I2E1$ model fit, but it does appear in the Voyager 2 (V2) $I2E1$ fit (the spatial offsets of an equivalent tilted dipole derived from the $I2$ coefficients are listed at the bottom of Table 1). An $I2E1$ model fit to all of the Voyager observations results in reduced non-axisymmetric terms ($m \neq 0$) and a g_2^0 appropriate to a northward offset.

These results, and inferences on the axisymmetry of Saturn's magnetic field drawn from the charged particle observations at Saturn^{12,13}, and current models of Saturn's interior¹, motivated a conceptual model and inversion technique which favours zonal ($m=0$) harmonics over tesseral ($n > m > 0$) and sectorial ($n=m$) harmonics. We accomplish this by introducing a renormalization of the model parameters $\bar{g}_n^m = g_n^m/100$ ($m \neq 0$). This is equivalent, in our search for a minimum length solution to the least-squares minimization, to *a priori* knowledge of the uncertainties of the parameter estimates¹⁴. This technique has the advantage that it allows non-axisymmetric components ($m \neq 0$) of the model to be introduced after the zonal harmonics to assess their importance. Details regarding the method of constructing solutions by sequential introduction of (independent) parameters can be found in refs 11, 15.

The results of these 'constrained' model fits are summarized in Table 1. Each solution uses only 3 of the 15 internal field parameters of an $I3E1$ spherical harmonic expansion since we found that inclusion of non-axisymmetric internal field terms offered minimal improvement in the model fits. Analysis of the V1 and V2 observations individually in terms of a physical model consisting of zonal harmonics to the order of $n=3$ leads to very consistent results. That is, the V1 model does an excel-

lent job of predicting the magnetic field observed by V2 in a very different region of space, and vice versa. Thus, this third-order zonal harmonic model behaves as a proper model whereas models including dipole and quadrupole terms only do not.

We therefore propose as a model of Saturn's planetary magnetic field the third-order zonal harmonic model fitted to both V1 and V2 observations, listed in Table 1 and hereafter designated as the ' Z_3 ' model (zonal of order 3). No equivalent offsets are listed for the axisymmetric models because the relative magnitudes of the derived g_n^0 terms are inconsistent with a simple axially offset of the dipole. The ratio g_3^0/g_2^0 is too large by a factor of ~ 30 to be attributed to a dipole offset.

The Z_3 model is an extremely efficient representation of Saturn's planetary field, requiring only three parameters. It fits the combined V1 and V2 observations with little more than half the r.m.s. residual of a best-fitting axially offset dipole. Inclusion of a (small) fourth-order zonal harmonic (g_4^0) does not significantly improve the fit to the observations nor does it alter appreciably the values of the Z_3 model parameters. Much of the remaining difference (~ 3 nT r.m.s.) between the Z_3 model magnetic field and the V1 and V2 observed field is due to external sources (in addition to and distinct from the ring current, which itself varied between V1 and V2 observations) which are not well modelled by the uniform external field approximation used here.

The Voyager 1 observations suggest the presence of a large-scale current system, the exact geometry of which is as yet undetermined but is thought to involve $\sim 6 \times 10^6$ A of polar current flowing into Saturn's south pole. The unmodelled external field observed during the Voyager 2 encounter appears to be attributable to solar wind induced time variations in Saturn's magnetosphere. A better understanding of these external sources will probably be required before the Z_3 model can be improved, perhaps with the inclusion of (relatively small) non-axisymmetric terms. In its present form, however, the Z_3 model is consistent with the Pioneer 11, Voyager 1 and Voyager 2 magnetic field observations as well as the charged particle micro-absorption feature observed by the Voyager 2 CRS investigation¹³ and identified with the satellite Tethys.

The Z_3 model leads to significantly enhanced polar surface field intensities on Saturn as shown in Fig. 1. Compare the surface field intensity of the Z_3 model as a function of latitude with that of an offset dipole model obtained by setting g_3^0 of the Z_3 model to zero, and that of a centred aligned dipole of moment $0.21 \text{ G} \cdot R_s^3$. The maximum polar intensities in the north and south are ~ 0.84 and ~ 0.65 G, comparable with the Earth's ~ 0.7 G polar intensities. Note also that the equatorial surface field magnitude is reduced to ~ 0.18 G by the large g_3^0 term of the Z_3 model. The relative magnitude of the Z_3 model g_n^0 coefficients is compared in Fig. 2 with the relative magnitude of the Earth's axisymmetric g_n^0 terms¹⁶. Saturn's g_n^0 are larger in magnitude than those of the Earth.

Cowling's theorem¹⁷ excludes the possibility of maintaining a steady state axisymmetric dynamo by fluid motions. It is possible, though improbable, that Saturn's highly axisymmetric field configuration is reflective of the decay phase of a magnetic field reversal¹⁸. More appealing is the suggestion that the dynamo itself is not axisymmetric¹ but only outwardly appears as such. If what we observe at Saturn is but the axisymmetric part of a complex Earth or Jupiter-like dynamo, the magnitude of Saturn's g_n^0 terms may be the only available observable relevant to Saturn's dynamo. The differential rotation of the metallic conducting shell above Saturn's dynamo in Stevenson's model¹ leads to an increase in the axisymmetric toroidal field deep within Saturn's interior. Some form of flux expulsion must inevitably occur to relieve the ever-increasing magnetic pressure. Could the process of flux expulsion result in a relatively short-lived and localized magnetic field anomaly unobserved by more distant spacecraft but sufficient to modulate Saturn's radio emission¹⁹? The lack of any detectable departure from axial symmetry remains the most enigmatic aspect of Saturn's unique magnetic field.

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Macroscopic evidence of molecular chirality in columnar mesophases

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Besides the classical liquid crystals observed with rod-like molecules (nematic and smectic mesophases) and plastic crystals obtained in the case of globular compounds, two new classes of thermotropic mesophases consisting of two-dimensional aromatic flat molecules have been investigated^{1–3}: D columnar mesophases (columns of disk-like molecules in regular two-dimensional periodic array) and N_D nematic mesophase (disk-like molecules parallel to an average plane). We report here two examples of molecular chirality directly observable in D mesophases of a resolved disk-like compound.

Some hexasubstituted benzene¹, triphenylene^{4–10}, truxene^{11–14} or anthraquinone^{15,16} derivatives (Fig. 1) exhibit highly viscous and birefringent mesophases between the solid state and the isotropic liquid, which correspond to a columnar arrangement of diskoid molecules.

These columnar structures, denoted D in Fig. 2a legend, differ from one another by the symmetry of the two-dimensional lattice and by the stacking, regular or not, of

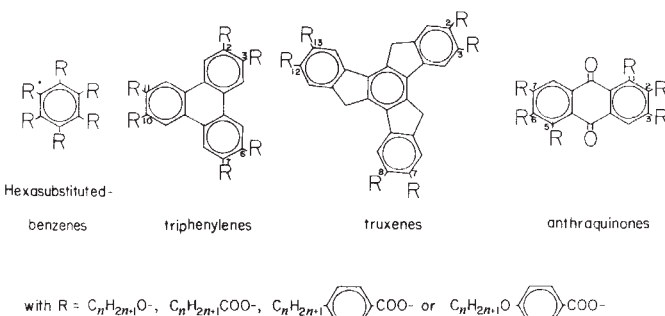


Fig. 1 Mesogenic disk-like cores.

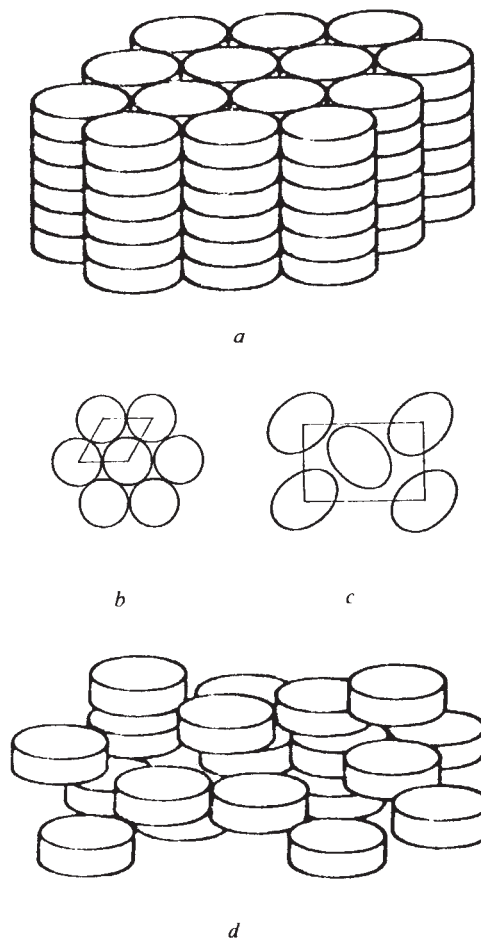


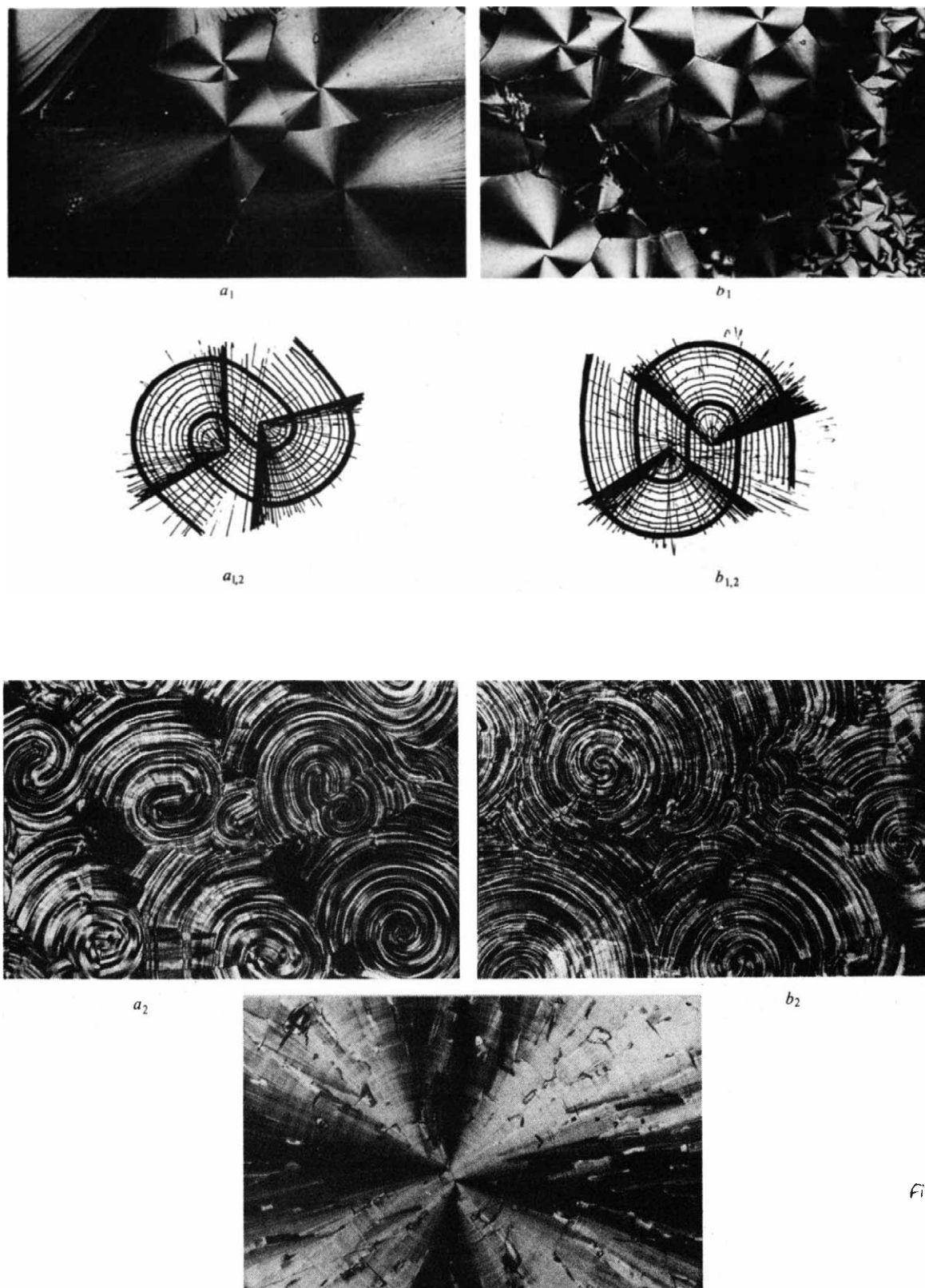
Fig. 2 a, Structure of columnar phase (with ordered disks). Schematic representation of hexagonal (b) and rectangular (c) phase lattices. D_{ra} phases are generally of the Pgg symmetry¹⁷, but one example of Pmg symmetry has been described^{15,16}. d, Structure of N_D nematic phase. The nematic nature of this phase has been checked by X-ray diffraction measurements⁹.

disk-like molecules in the columns²: hexagonal two-dimensional lattices (Fig. 2b) with order (D_{ho}) or disorder (D_{hd}) of the molecules in each column, rectangular two-dimensional lattice (Fig. 2c) with disorder of the molecules in each column (D_{rd}). The columns can be upright or tilted with respect to the molecular reference plane. A single example of strongly tilted columnar phase (D_t) has been observed (tilt angle ~55°)⁹.

A fluid phase (N_D) with optical schlieren texture, in any point similar to the classical nematic one, forms the second class of mesophases exhibited by disk-like compounds. This N_D nematic phase, in which the only order is a parallelism between flat cores (Fig. 2d), is observed with several hexa (*p*-alkyl or alkoxy) benzoyloxytriphenylenes^{7–10} or truxenes¹⁴ and with some hexaalkanoxytruxenes^{11–13}. The chiral variant, cholesteric (N_D^{*})^{18,19}, is obtained with optically active diskoid molecules.

We have recently described the two mesogenic enantiomers of the hexasubstituted triphenylene (I)¹⁸. Our first observations of the two successive thermotropic columnar mesophases of (+) or (–) (I) mounted between two glass slides and viewed under a polarizing microscope did not reveal any chirality in the medium. Nevertheless, we have shown that these substances could easily twist the N_D nematic phase of some hexabenzoyloxytriphenylenes as might be expected. Furthermore, we recently reported the existence of a N_D^{*} cholesteric phase for a pure disk-like compound¹⁹.

The large analogy between columnar and smectic phases had given us an inkling of the existence of a twisting induced by



Fig

Fig. 3 *a*, (+)-(*I*); *b*, (−)-(*I*); *c*, (±)-(*I*). *a*₁ and *b*₁, enantiomorphic 'opposite points' in the *D*_{r1} phase (×100). *a*_{1,2} and *b*_{1,2}, enantiomorphic 'opposite points' and spirals at *D*_{r1}→*D*_{r2} transition (schematic representations). *a*₂ and *b*₂, enantiomorphic spirals in the *D*_{r2} phase (×100). *c*, Racemic texture at *D*_{r1}→*D*_{r2} transition (detail). The two columnar phases of (*I*) were first identified with *D*_{rd} and *D*_{hd} owing to their textures and the miscibility of the low-temperature mesophase with some *D*_{rd} phases of hexaalkanoxytriphenylenes. But recent X-ray diffraction results on one enantiomer point out that the high-temperature columnar phase is, in fact, another rectangular phase with different symmetry. Pending more refined X-ray diffraction measurements we shall name the high temperature phase: *D*_{r1} and the low temperature one: *D*_{r2} (A. M. Levelut provided these preliminary data). (+)-(*I*) and (−)-(*I*) were purified by thin-layer chromatography on silica gel (using 10% acetone/hexane mixture as eluant) and recrystallized from ethanol/ether mixture. (+)-(*I*): [α]₂₇₈²⁵ = +12.4° (c, 1 in chloroform) (Found: C, 74.70; H, 9.71; C₇₈H₁₂₀O₁₂ requires C, 74.96; H, 9.68). (−)-(*I*): [α]₂₇₈²⁵ = −11.6° (c, 1 in chloroform) (Found: C, 74.81; H, 9.84; C₇₈H₁₂₀O₁₂ requires C, 74.96; H, 9.68). IR (neat) 1,770; 1,515; 1,255; 1,200; 1,125; 920 cm^{−1}. ¹H NMR (CDCl₃) δ 0.9 (s, 18 H), 1.1 (d, *J* = 6.3 Hz, 18 H), 1.35 (s, 60 H), 2.1 (m, 6 H), 2.5 (m, 12 H), 8.2 (s, 6 H).



Fig. 4 Spiral column (with disordered optically active molecules).

optically active molecules in a tilted columnar phase (D_1)¹⁸, as was the case with biaxial tilted smectics (S_C , S_F , S_I) composed of rod-like molecules^{20,21}.

The two columnar phases of (*I*) were first described as upright D_{rd} and D_{hd} phases¹⁸; consequently the absence of twisting with optically active solute molecules looked 'normal'. But some recent optical observations—in the case, for example, of (*I*) with $R = n$ -alkanoyl chain (P. Oswald, personal communication)—seem to favour a slight tilt of the columns, and so we have made new observations on free droplets.

A careful re-examination of the two columnar mesophases of compound (*I*) allowed us to observe an evident chirality and to prove the existence of new chiral mesophases.

The transition temperatures (°C) of (+) or (−) (*I*) are:

K 59 D_{r2} 64.5 D_{r1} 97.5 I (K, crystal; I, isotropic liquid).

The racemic mixture (±) (*I*) melts at 47.5 °C and the isotropic phase appears at 95.5 °C.

On cooling isotropic free droplets, one can see—between crossed polarizers—near 96 °C, a fan-shaped texture consisting of only one type of pairs with 'opposite points'. Both enantiomers exhibit similar non-superposable figures, each one being the reflection image of the other (Fig. 3*a*₁, *b*₁). On further cooling, near the $D_{r1} \rightarrow D_{r2}$ transition, a spiralization of the medium is evident: some spirals are generated around the pairs of opposite points as shown in Fig. 3*a*_{1,2}, *b*_{1,2}. On heating immediately after the spiralization takes place, the effect remains up to the clarification point, but if the temperature is

maintained for a few minutes a progressive change in the spiral figures can be seen. Once more we observe two antipodal figures (Fig. 3*a*₂, *b*₂). In addition the spirals reveal a periodicity of the oily streaks (note that, in all the figures, 'opposite points' and spirals are visible—but less clearly—between uncrossed polarizers).

In the case of an equimolecular mixture of (+) (*I*) and (−) (*I*), we observe, as expected, non-chiral figures resembling concentric rings (D_{r2}) or face-to-face 'opposite points' (D_{r1}) (Fig. 3*c*). We have noticed that many achiral disordered columnar phases ((*I*) with $R = n$ -alkanoyl chain) consist of a statistical mixture of right- or left-handed spirals or opposite points in addition to some perfect achiral figures.

3-Methylnonanoic acids (R) (−) (*IV*) and (S) (−) (*IV*) have been prepared by a convenient adaptation of a procedure described by Prout *et al.*²² from (S) (−) and (R) (−) 2-octanols (via the *p*-toluenesulphonates). Hexaesters (+) (*I*) and (−) (*I*) were obtained by interaction of the 3-methylnonanoyl chlorides (respectively from (R) (−) (*IV*) and (S) (−) (*IV*) with 2, 3, 6, 7, 10, 11-hexahydroxytriphenylene (*II*) prepared by heating the corresponding hexamethoxy derivative (*III*) with pyridine hydrochloride²³. Compound (*III*) was prepared by treating veratrole with *p*-chloranil in sulphuric acid²⁴.

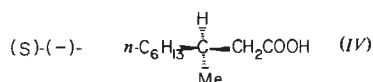
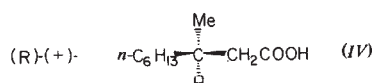
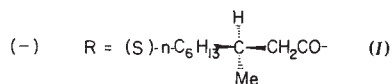
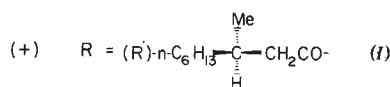
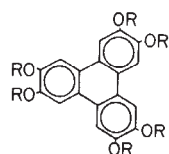
We have presented the first evidence of two rectangular columnar mesophases which form examples of a directly observable molecular chirality. Nevertheless, the chiral molecular packing in these media raises several questions:

- Why are the enantiomorphic figures clearly visible only on free droplets?
- Is each column chiral—as, for instance, one of the enantiomorphic spiral columns of St Peter's high altar in the Vatican City (Fig. 4)?
- Or is this phenomenon connected to a peculiar chiral packing on the surface of the mesomorphic droplet?

We are now carrying out optical studies and X-ray diffraction measurements to understand more closely the structure of these two mesophases.

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Electron density images from imperfect data by iterative entropy maximization

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Information theory provides a uniquely powerful apparatus for reconstructing an image from imperfect data in its dual space. An iterative procedure based on constrained entropy maximization is presented here for reconstruction of electron density from imperfect single-crystal X-ray diffraction data. In the ideal situation, continuous electron density, its periodicity given by the lattice, is the Fourier transform of an infinite set of structure factors whose squared moduli are observables. An ideal electron density is unattainable from experiment because of the common experimental inadequacies of incomplete and noisy data discussed by Gull and Danielli¹. These inadequacies are partially overcome in the present procedure which yields true super-resolution and is economical even for protein crystal structures as illustrated.

Although Gull and Danielli¹ focused on radioastronomical applications, their maximum entropy solution for a best map of radio intensity from experimental data is quite general, and its formalism is appropriate to the X-ray crystallographic problem. Nevertheless, the formalism is unsatisfactory for many crystallographic applications on two counts. First, convergence properties are not characterized and appear to be undesirable as recourse to partial sums was required for convergence in the reported application¹. Second, the formalism fails in the absence of noise; this is a common difficulty with many spectral analyses based on entropy maximization². It could be argued that artificial introduction of noise is possible in the presumably few applications for which the naturally occurring noise level is inadequate. However unlikely, such a state of affairs in experiments carefully designed to minimize or offset natural noise constitutes a dilemma to be avoided.

A formalism, designed from the outset to result in an iterative procedure, for a maximum-entropy mapping of electron density can be readily composed with the aid of an approximation for the natural logarithm. We assume that the corresponding inexactness for the most part becomes immaterial on iteration as in any successive approximation procedure. It also will be assumed that the density functions and the experimental data are discrete samplings on regular grids appropriate to discrete Fourier transform algebra³.

Consider that there is a positive definite trial electron density τ obtained from the transform of experimental structure factors $\hat{F}$ (experimental modulus, model-based phase); the scale of $\hat{F}$ is set to make $\hat{F}_{k=0}$ equal the number of electrons in a unit cell. Within the range of observations it is desired that F the transform of ρ differs from $\hat{F}$ as little as possible subject to maximization of the entropy which measures the difference between ρ and τ , ρ constrained to be positive definite. After the pattern of Gull and Danielli¹ maximize

$$Q(\lambda) = -\sum_{\mathbf{x}} \rho'_{\mathbf{x}} \ln [\rho'_{\mathbf{x}}/\tau'_{\mathbf{x}}] - \frac{\lambda}{2} \sum_{\mathbf{k} \in K} |F_{\mathbf{k}} - \hat{F}_{\mathbf{k}}|^2 / \sigma_{\mathbf{k}}^2$$

Table 1 The course of reconstruction

	At the start	After four iterations
R	0.22	0.14
$\langle \alpha_{\text{IR}} - \alpha_{\text{MEM}} \rangle^*$	0 (0)	25 (32)
$\langle \alpha_{\text{LS}} - \alpha_{\text{MEM}} \rangle^*$	36 (44)	31 (38)

Tabulated quantities are explained in the text.

* Modulus weighted averages are given; the corresponding un-weighted averages are given in parentheses. All values are in degrees.

by setting

$$\partial Q(\lambda) / \partial \rho_{\mathbf{x}} = 0$$

where the sum over $\mathbf{x}$ includes a complete unit cell, the entropy formula⁴ uses $\rho' = \rho / \sum \rho_{\mathbf{x}}$ and $\tau' = \tau / \sum \tau_{\mathbf{x}}$, λ is a disposable constant to be evaluated, K denotes the set of reciprocal lattice points for which there are useful observations, and σ is the standard deviation of $\hat{F}$. Evidently

$$-\ln \rho_{\mathbf{x}} + \ln \tau_{\mathbf{x}} + A + \hat{F}_0 \lambda \sum_{\mathbf{k} \in K} \frac{1}{\sigma_{\mathbf{k}}^2} (\hat{F}_{\mathbf{k}} - F_{\mathbf{k}}) \exp \{-2\pi i \mathbf{k} \cdot \mathbf{x}\} = 0 \quad (1)$$

for which the relationship $\hat{F}_0 = \sum (\text{volume/grid point}) \rho_{\mathbf{x}}$ has been used, it is assumed that $-\mathbf{k} \in K \forall \mathbf{k} \in K$, and A is given by

$$A = (\sum \rho_{\mathbf{x}})^{-1} \sum \rho_{\mathbf{x}} \ln [\rho_{\mathbf{x}}/\tau_{\mathbf{x}}]$$

If as desired the agreement between $\hat{F}$ and F is good, then A which measures the scale disparity between ρ and τ is small. Neglect of A and rearrangement of equation (1) result in

$$\rho_{\mathbf{x}} = \exp \left[\ln \tau_{\mathbf{x}} + \hat{F}_0 \lambda \sum_{\mathbf{k} \in K} \frac{1}{\sigma_{\mathbf{k}}^2} (\hat{F}_{\mathbf{k}} - F_{\mathbf{k}}) \exp \{-2\pi i \mathbf{k} \cdot \mathbf{x}\} \right] \quad (2)$$

which forms the basis for the reconstruction procedure presented here. Note that equation (2) certainly satisfies one of the two earlier mentioned problems of convergence and (possibly) noise-free data. Disappearance of the latter problem is trivially evident, for if the modelled density should ever transform to $F_{\mathbf{k}} = \hat{F}_{\mathbf{k}}$, $\forall \mathbf{k} \in K$, then $\rho = \tau$ and cannot be changed by use of equation (2). Although neglect of A may bias the entropy term, a formalism results which, as desired, does not fail in the absence of noise. On the other hand, use of $A = 0$ means the convergence question must await an empirical answer as initially provided in the illustration.

Equation (2) may be rearranged to yield

$$\ln [\rho_{\mathbf{x}}/\tau_{\mathbf{x}}] = \hat{F}_0 \lambda \sum_{\mathbf{k} \in K} \frac{1}{\sigma_{\mathbf{k}}^2} (\hat{F}_{\mathbf{k}} - F_{\mathbf{k}}) \exp \{-2\pi i \mathbf{k} \cdot \mathbf{x}\}$$

For good agreement of ρ and τ the logarithm may be approximated as

$$\ln x \approx x - 1$$

to give

$$(\rho_{\mathbf{x}} - \tau_{\mathbf{x}}) = \tau_{\mathbf{x}} \hat{F}_0 \lambda \sum_{\mathbf{k} \in K} \frac{1}{\sigma_{\mathbf{k}}^2} (\hat{F}_{\mathbf{k}} - F_{\mathbf{k}}) \exp \{-2\pi i \mathbf{k} \cdot \mathbf{x}\}$$

which permits a formal estimation of λ at τ_{max} . Correspondence with standard crystallographic difference map formalism⁵ implies for λ the evaluation

$$\lambda = (V \tau_{\text{max}} \hat{F}_0)^{-1} \left(\frac{1}{n_K} \sum_{\mathbf{k} \in K} \frac{1}{\sigma_{\mathbf{k}}^2} \right)^{-1} (2 - \delta_1)$$

where V is the volume of a unit cell, n_K is the number of terms in the sum, and $\delta_1 = 1$ or 0 according to the presence or absence of a centre of symmetry. For constant errors, $\lambda = (V \tau_{\text{max}} \hat{F}_0)^{-1} \sigma^2 (2 - \delta_1)$. It follows that the standard crystallographic difference synthesis may be interpreted as an approximate difference between unit-weighted maximum-entropy representations of experimental and modelled electron densities.

Deterministic interpretation of the logarithm of equation (2) suggests the form

$$\ln \rho_{\mathbf{x}} = \ln \tau_{\mathbf{x}} + \text{corrective terms}$$

or

$$\ln (\rho_{\mathbf{x}}/\tau_{\mathbf{x}}) = \text{corrective terms}$$

For a formal difference of $<10\%$ between τ and ρ , the left-hand side may be approximated within 0.1% as

$$\ln (\rho/\tau) = (\rho - \tau)/\frac{1}{2}(\rho + \tau) \approx \Delta\rho/\rho$$

Evidently this would be an accurate deterministic form for the

corrective terms. With λ evaluated at $\tau_{\max}$, the maximum entropy solution for corrective terms as given in equation (2) differs from the deterministic form only as the values for ρ move down from their highest levels. This fortuitously corresponds with the experimental situation in which the lowest levels of a density function are usually rather less significant than the highest levels. Relative to accurate deterministic corrections, the maximum-entropy corrections down-weight the significance of the lowest density levels through absence of the ρ^{-1} dependence. Moreover, because the highest density levels contribute most to the value for F , and because the maximum entropy corrections place functional emphasis on correcting the highest density levels, it is expected that repeated application of equation (2) quite generally will constitute a stable, convergent, iterative reconstruction of electron density. Although in the presence of error the resulting density may never be better than an asymptotic approximant, it will be positive definite, well behaved, and in good agreement with the observations by the criterion of a constrained maximum of configurational entropy.

'Super-resolution' is a term used^{1,2} to denote the fact that maximum-entropy methods can yield functions of resolution higher than that which corresponds to the band limits of the data. The term is sometimes used to indicate that the presence of noise in a set of data can lead to apparent but improperly high resolution. Super-resolution of the proper sort is highly desirable and can be understood to arise in the present case through imposition of a (perfectly general) model for electron density. While the maximum-entropy formalism yields a result which is maximally non-committal with regard to missing or unavailable data^{6,7}, it does require an exponential representation for the density function. Because the sampling of the density may be made arbitrarily dense, the exponential representation corresponds to a function which is not only positive definite and well behaved, but also continuous and consequently possessed of an infinite transform with unlimited resolution. There is no general way to know how well the implied transform samples for $\mathbf{k} \notin K$ agree with the unavailable observations or even that they agree at all. But in crystallographic application maximum-entropy formalisms can lead to density maps at more

than twice the resolution expected for straightforward Fourier synthesis of structure factors⁸. As we now show, iterative reconstruction based on equation (2) does in fact produce real super-resolution.

The rubredoxin of *Clostridium pasteurianum* has been studied in single-crystal X-ray diffraction experiments and its noncentrosymmetric structure reported⁹ at a resolution of 1.5 Å (minimum interplanar spacing). A structure model was fitted to the 1.5 Å experimental data by least squares and the structure-factor phases computed from this model, the experimental structure-factor moduli, and the atom coordinates of the model are all given in the structure report⁹. Phases which had been determined by isomorphous replacement methods at 2.0 Å resolution were provided by Dr Sayre with permission from Professor Jensen. The reconstruction procedure used for the present illustration may be outlined as follows. (1) Compute an electron density τ using $\hat{F}$ the experimental structure moduli and phases from isomorphous replacement, all at 2.0 Å resolution, and to ensure a positive definite density at the start replace τ by the larger of (τ , $\tau_{\max}/100$) for each position $\mathbf{x}$. Compute and file $\ln \tau$ and the transform of (modified) τ as initial F . (2) Computation of equation (2) with the phase of F assigned to $\hat{F}$ gives ρ whose transform is a new F and logarithm is a new $\ln \tau$. (3) Scale $|F|$ so it is on the same scale as $|\hat{F}|$ and compute

$$R = \sum_{\mathbf{k} \in K} \frac{||\hat{F}_{\mathbf{k}}| - |F_{\mathbf{k}}||}{\sum_{\mathbf{k} \in K} |\hat{F}_{\mathbf{k}}|}$$

(4) Repeat steps (2) and (3) until the improvement in R is judged to be not significant.

Although 1.5 Å data are available for rubredoxin, only the 2.0 Å subset together with initial isomorphous-replacement phases were used in the reconstruction. This allows for demonstration of super-resolution by comparison of the reconstructed density function with a reference density using about twice as much data. The reference density was obtained by standard Fourier synthesis of a complete 1.5 Å structure-factor set obtained as experimental moduli and phases calculated from the refined atomic model; these calculated phases are considered to be correct for this illustration.

The course of the calculation through four iterations is summarized in Table 1. Each iteration is based on a forward and reverse Fourier transformation¹⁰ of samples on a grid of 64 points per cell edge. Computing time for each complete iteration was ~30% more than required for the Fourier syntheses alone. For the 2,812 unique data used, the average magnitude of difference between the phases from the maximum entropy method α_{MEM} and the phases from the atomic model, the so-called least-squares phases α_{LS} , is given in Table 1. A companion entry gives the average magnitude of difference between α_{MEM} and the phases from isomorphous replacement α_{IR} . These average phase deviations taken together show that the maximum entropy method yields a result which in some sense concerning phases lies between the sets from isomorphous replacement and least-squares refinement. The value for $R = \sum ||\hat{F}| - |F|| / \sum |\hat{F}|$ is amenable to more definite interpretation. A first significance of R is as a criterion of convergence for the reconstruction procedure. An analytical discussion of this point is lacking but it is easy to see that the procedure will stop if and only if $R = 0.0$. Yet if R stops changing between iterations, then clearly in some average sense no further desirable change is occurring. A second use for R or its value at convergence is as a preliminary indicator of global error in a data set. Because the exponential model for electron density is perfectly general, error-free data ideally should yield $R = 0.0$ at the conclusion of a maximum-entropy reconstruction. Of course, data are not error-free and random data errors correspond to an uncorrelated, necessarily zero-mean process which is not representable by an exponential model. The maximum entropy procedure may, nevertheless, offset some error effects, but in unpredictable ways because the exponential representation of density plus error destroys the additive relationship. With reservation

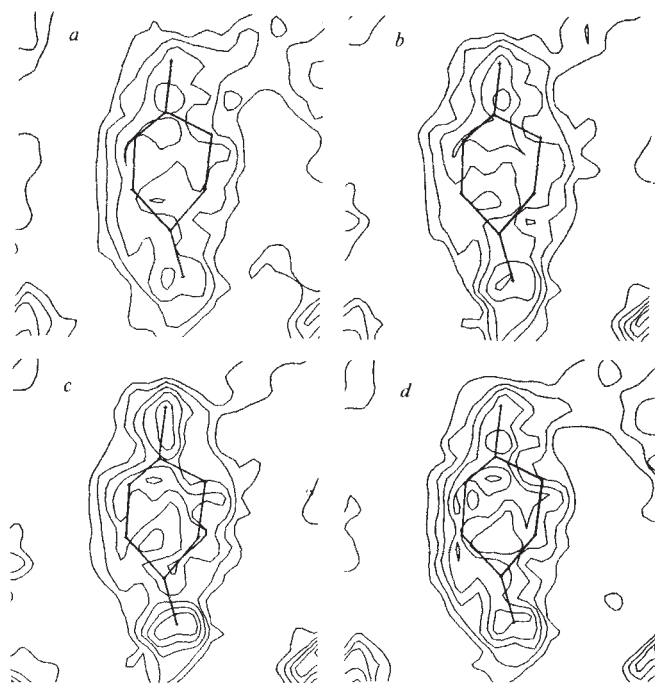


Fig. 1 a, Tyrosine-11 based on 2.0 Å data and isomorphous replacement phases. b, Tyrosine-11 based on 2.0 Å data and least-squares phases. c, Tyrosine-11 based on 1.5 Å data and least-squares phases. d, Tyrosine-11 in a maximum entropy reconstruction iteratively developed from the data and phases used in a.

in this regard and also concerning the possibility of false entropy maxima, it seems that the asymptotic value of R should approximate $\sum|\epsilon|/\sum|\hat{F}|$, ϵ being the true experimental error in $|\hat{F}|$.

Quality of image is demonstrated in Fig. 1 by comparison of different representations of the tyrosine-11 residue of rubredoxin⁹. Tyrosine-11 was chosen on the joint grounds of its planarity and previous use as an example^{9,11}. Figure 1a–d shows interpolated sections of density maps in the least-squares plane of the reported coordinates for atoms in the planar tyrosine group. Contour levels begin at 0.0 and increase in steps of 0.30 electrons $\text{\AA}^{-3}$; the line segment ends correspond to reported atomic positions. Figure 1a is based on 2.0 $\text{\AA}$ data with phases from isomorphous replacement and represents the starting point for all further structure description and refinement. Figure 1b, c are based on the least-squares phases calculated from the atomic model. Although the reported atomic model and associated phases are being used here as a kind of standard, note that in the original report they were considered subject to improvement. Clearly, Fig. 1b which is based on 2.0 $\text{\AA}$ data and least-squares phases gives a better representation of tyrosine than does Fig. 1a. Figure 1c which is based on least-squares phases and the complete 1.5 $\text{\AA}$ data set is even better and of obviously higher resolution. The maximum-entropy reconstruction is given in Fig. 1d which is more similar to Fig. 1c than to the others. In fact one could reasonably argue from their appearance that Fig. 1d, if anything, is of higher resolution than Fig. 1c. Clearly, the maximum entropy reconstruction based on only 2.0 $\text{\AA}$ data and isomorphous-replacement phases shows real super-resolution as it is of obviously greater true resolution than the classical representation of the same data given in Fig. 1a.

Maximum-entropy methods for image reconstruction potentially yield the best image model derivable from the available data². This does not refer to a best image model in an absolute sense, but in the sense of being the model least likely to need correction on measurement and consideration of additional data². While the virtues of maximum entropy analysis are claimed for the present procedure, the approximations used mean that its details could be improved on. In connection with the rubredoxin illustration, note that individual standard errors for structure moduli were not used and the default unit values are not commensurate with optimum results from the calculations. Even so, in contrast to other types of image enhancement^{8,12}, the present procedure seems to be exceptionally stable and requires no special adaptation for dominating atoms or groups as, for example, the iron-sulphur group of rubredoxin. While no evidence has been developed in specification of a radius of convergence, it seems that the outlined procedure is applicable without further modification in every case for which there are structure moduli and corresponding initial phases of the quality normally attained by isomorphous replacement methods.

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Structure of the Red Sea at 20° N from gravity data and its implication for continental margins

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Recent gravity data have enabled a gravity profile extending 1,500 km over Nubia, the Red Sea and Arabia to be compiled. Using a modal value of -48 mGal for the Bouguer anomaly over Africa away from regions of rifting we have determined the way in which the lithosphere thins and breaks to form the Red Sea. We find that nearly the whole of the Red Sea from coast to coast is probably underlain by oceanic crust and the regions of thinning extend for ~120 km landwards of the Nubian coast and 180 km landwards of the Arabian coast. This implies that for continental margins similar to those of the Red Sea, the vast thicknesses of sediments might be underlain by early oceanic lithosphere rather than by thinned and down-faulted continental lithosphere.

The Red Sea is a critical region for studying the breakup of the continents, the evolution of continental margins and new oceanic crust. The presence of large positive Bouguer gravity anomalies over the deep axial trough suggested that the trough might be underlain by dense oceanic crust¹. This was confirmed in the 1960s when high seismic velocities and large magnetic anomalies were found². The magnetic anomalies were interpreted in terms of seafloor spreading³ and a model constructed in which the deep axial trough was underlain by oceanic crust formed over the past few million years and the main trough and shelves were underlain by downfaulted shield rocks covered by large thicknesses of sediments^{4,5}. In 1970 McKenzie *et al.*⁶ applied plate tectonics and fitted the remarkably parallel shorelines (previously noticed by Wegener). This stimulated much new work on the nature of the crust underlying the vast thicknesses of sediments to the sides of the axial trough. In particular, magnetic surveys showed remarkable lineations and the suggestion was made that the Red Sea may have formed in at least two stages of seafloor spreading⁷. Further studies⁸ showed magnetic lineations over the margins of the Red Sea to be widespread. Recently, a very detailed aeromagnetic survey (R. Horn, personal communication) has been carried out between Sudan and Arabia on behalf of the Red Sea Commission. This shows extensive lineations with remarkable symmetry. The magnetic anomalies thus suggest the presence of oceanic crust beneath the sediments and extending approximately to the coasts at least in the southern two thirds of the Red Sea. This is also supported by the interpretation of the gravity anomalies.

Gravity data from a recent geotraverse across northern Africa⁹ enable a long profile to be constructed across the Red Sea and its neighbouring Nubian and Arabian plates. The location of the profile is shown in Fig. 1. The coordinates of the stations in Nubia and Arabia were projected onto a line with azimuth N56° passing through Port Sudan. For Nubia, 32 stations obtained on the geotraverse provided simple Bouguer anomalies and heights at an average spacing of 16 km. For the Red Sea, Bouguer anomalies and depths on a N56° line from Port Sudan to Jeddah were obtained from charts with contour intervals 10 mGal and 100 m respectively¹⁰. For Arabia, Bouguer anomalies and heights for 21 stations¹¹ eastwards from the Red Sea Arabian coast at an average spacing of 15 km were used. The complete profile (Fig. 2a) shows that the whole of the Red Sea from coast to coast is associated with positive

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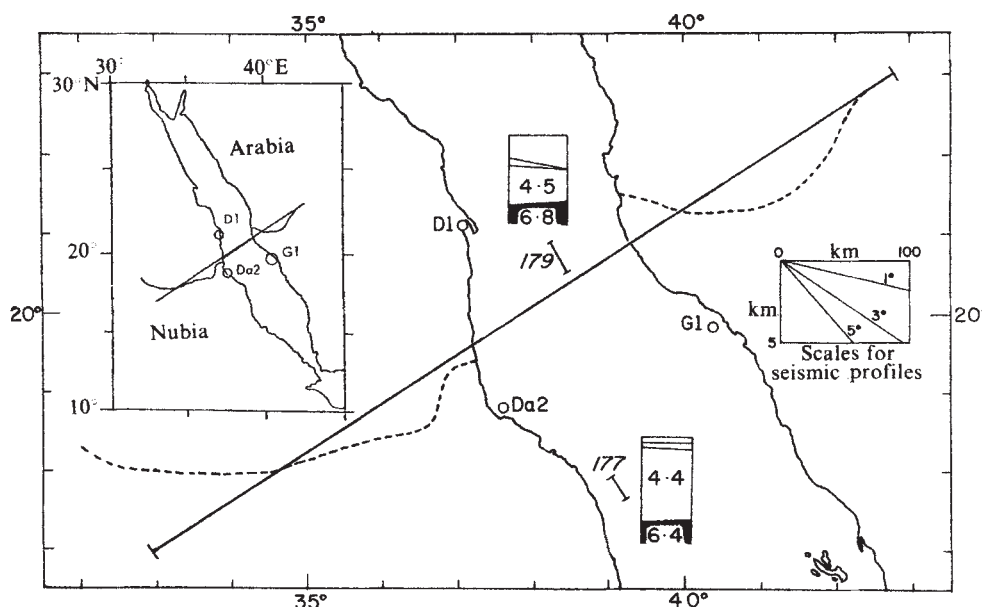


Fig. 1 The location of the profile and its projection on to the line N56°. The locations of the bore-holes Dungunab-1 (D-1), Durwara-2 (Da-2) and Ghawwas-1 (G-1) and seismic refraction lines 177 and 179 are also shown.

Bouguer anomalies which reach a maximum of more than +100 mGal. Landwards from the coasts the anomalies become negative reaching values of -50 mGal on the Nubian side and -100 mGal on the Arabian side.

To interpret the profile we need to make assumptions concerning the thickness and structure of the lithosphere away from the regions of rifting and to estimate the regional gravity anomaly. For the continental US, isostatic response data¹² seem to be incompatible with density anomalies below ~80 km but in Canada observations of the wavelength and amplitude of bending in the vicinity of supercrustal loading¹³ give estimates of ~110 km for lithospheric thickness. For unrifted Africa, the simple assumption is made that the lithosphere is 100 km thick and is associated with a Bouguer anomaly of -48 mGal derived from a statistical study of $1^\circ \times 1^\circ$ means¹⁴. The structure of the African lithosphere based on seismic studies¹⁵ with the velocities converted to densities using the Nafe-Drake curve¹⁶ is shown in Fig. 2a and the more detailed structure of the crustal part in Fig. 2b.

For the continental part of the profile it is necessary to compute geological corrections for the light volcanics and sediments near Harrat Rahat and Harrat al Kishb at ~22.5°N, 41°E. A uniform specific gravity (SG) of 2.3 was assumed with the volcanic pile underlain by Precambrian basement at sea level. It is also necessary to correct for the light sediments on the Nubian and Arabian coastal plains. These were assumed to have a uniform SG of 2.2 with thicknesses taken from the Tectonic Map of Africa¹⁷.

For the Red Sea, there are three boreholes in the neighbourhood of the profile¹⁹, two on the Nubian side and one on the Arabian side (Fig. 1). All are close to the coasts. On the west side, the borehole to the north, at 21.10°N, 37.10°E (Dungunab-1) has 1,563 m of sediment and the borehole to the south at 18.81°N, 37.64°E (Durwara-2) has 4,152 m of sediment. Both then penetrated basalt. The borehole on the east side at 19.84°N, 40.37°E (Ghawwas-1) has a sediment thickness >3,446 m. There are also two reversed seismic refraction profiles² which constrain the velocity structure to the top of the oceanic layer 3 in the main trough (line 177) and axial trough (line 179). The velocities were converted to specific gravities using the Nafe-Drake curve¹⁶ giving values of 2.00 (equivalent to 2.3 km s⁻¹) for the sediments, 2.45 (4.4 km s⁻¹) for the evaporites and 2.80 (6.45 km s⁻¹) for oceanic layer 3. The Mohorovičić discontinuity was not detected on these lines and a depth of 11 km was assumed. If it is assumed that isostatic compensation is complete at 100 km (the depth assumed for the base of the African lithosphere¹⁴), a simple calculation shows that the average specific gravity of the oceanic mantle

beneath line 177 in the main trough is ~3.25. The details of the Red Sea structure used in the interpretation of the gravity profile are shown in Fig. 2b.

With these constraints the lithospheric structure was adjusted until the Bouguer anomaly was fitted to within ±10 mGal over the whole length of the profile (Fig. 2a).

The resulting structure shows the following features from west to east: (1) As the western margin of the Red Sea is approached, the lithosphere begins to thin rapidly. (2) The continent-ocean boundary is only 7 km seaward of the Nubian coast at this latitude. (3) A crustal intrusive zone about 90 km wide exists beneath the centre of the Red Sea. Its top is at a depth of 4 km and it has a SG of 2.88 corresponding to the 6.76 km s⁻¹ layer observed on seismic line 179. (4) The ocean-continent boundary coincides with the coastline on the Arabian side. (5) The region of lithospheric thinning has a slightly different form on the eastern side. (6) The 'normal' lithosphere is reached between 120 and 180 km from the coasts.

The model demonstrates that nearly all of the Red Sea at 20°N is underlain by oceanic crust but note that the exact demarcation of its boundaries depends critically on the specific gravities and thicknesses of the sediments and evaporites forming the marginal shelves.

A similar result has been obtained by Gettings¹⁹ for a gravity survey of the Arabian margin between 16.3°N and 17.3°N, which includes the Tihamah Asir Coastal plain. Here, an extremely steep gradient (4–5 mGal km⁻¹) is observed at the Tihamah-shield boundary. Gettings interprets this as indicating the presence of oceanic crust beneath the Tihamah as well as beneath the Red Sea shelf.

Independent support for the presence of oceanic crust beneath the Red Sea shelves (apart from the magnetic lineations discussed earlier) comes from the fact that the whole of the Red Sea is a region of high heat flow²⁰. It has been shown²¹ that the heat flow values from boreholes on the Sudan coastal plain are considerably higher than would be expected from the radioactive heat production of granitic continental crust.

Although our results are based primarily on the gravity profile across the Red Sea, they are consistent with other geophysical data for this region. The conclusion that the whole of the Red Sea at 20°N, including the vast thicknesses of sediments on the shelves is underlain by oceanic crust agrees with results from the westernmost Gulf of Aden²² where seismic reflection and gravity data require the ocean-continent boundary to be between the 183 m contour and the coast. In these regions, attenuation of the lithosphere, such as there is, seems to be landward of the coasts. Other passive continental margins similar to those of the Red Sea and Gulf of Aden might have

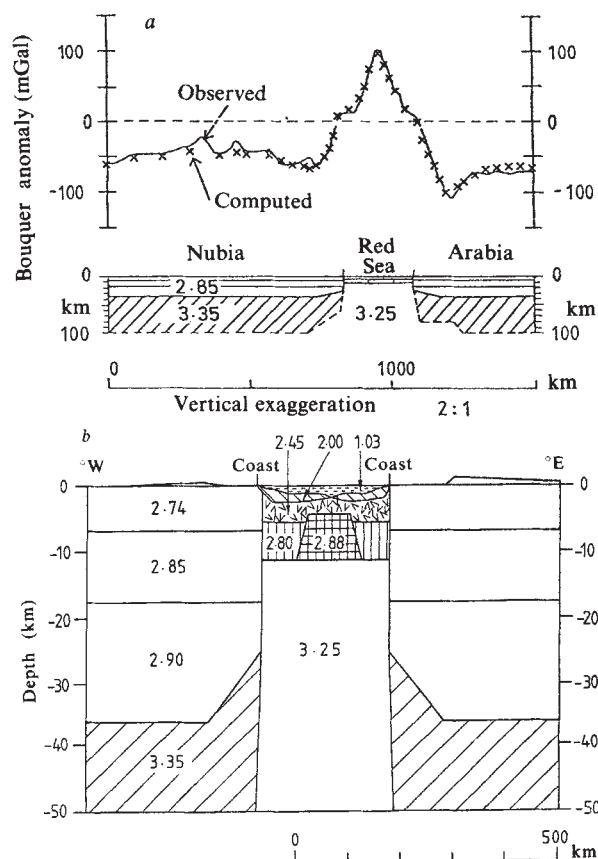


Fig. 2 *a*, The Bouguer gravity anomaly profile with the lithospheric structure used to fit the observed values. Details of the crustal structure of the Red Sea and the neighbouring continental lithosphere are shown in Fig. 2*b*. The positive anomaly over the Red Sea is due to the intrusive zone penetrating upper parts of the lithosphere giving a positive density contrast. The sharp boundaries between the anomalous mantle beneath the Red Sea and the normal mantle beneath the continents have been introduced to facilitate the gravity computations and should not be construed to represent an abrupt transition between geologically distinct regions in the mantle. *b*, Details of the structure of the crust and upper mantle used in the interpretation of the gravity profile shown in *a*. The attraction of each layer has been computed using a reduced specific gravity $\rho' = \rho - 2.67$ where ρ takes the following values: 2.00 (sediments); 2.45 (evaporites); 2.80 (oceanic layer 3); 2.88 (crustal intrusion); 3.25 (anomalous mantle) for the Red Sea and 2.74 (upper crust), 2.85 (middle crust); 2.90 (lower crust), 3.35 (normal mantle) for the continental lithosphere. The attraction of the mantle layers have been computed to a depth of 100 km.

oceanic crust underlying their shelves and we suggest that theories for the evolution of such 'continental margins' should consider this possibility.

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Significance of $^{87}\text{Sr}/^{86}\text{Sr}$ ratios in the Merensky cyclic unit of the Bushveld Complex

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In a number of mafic layered intrusions, layers with platinum group element (PGE) mineralization coincide with breaks in cumulus mineral composition and isotopic variation trends through the layered sequence^{1,2}. For example, in the Bushveld Complex in South Africa, abrupt increases in initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios occur at the UG-2 and Merensky pegmatoid (or 'reef') horizons, both of which are exploited for PGEs. We report here the results of a detailed Sr-isotope study across the Merensky unit and its immediate foot- and hangingwall-rocks which, when considered together with new ideas on processes operating in magma chambers^{3–5}, suggest that magma mixing and post-cumulus infiltration of liquids displaced from below were important during crystallization of the Merensky pegmatoid.

In the Bushveld Complex the PGE-bearing Merensky pegmatoid is part of the Merensky cyclic unit which occurs at the base of the main zone (subdivision after Willemse⁶—Fig. 1). Above the Merensky unit lies the lithologically similar Bastard cyclic unit. Both these units consist of a basal layer of orthopyroxenite which grades upwards through mela- and leuconorite into anorthosites with sporadically developed poikilophitic orthopyroxene crystals ~5 cm across, known as 'mottled anorthosites'. Thin stringers of chromitite usually occur at the base of each unit. In the case of the Merensky unit a layer of pegmatoid consisting mainly of large orthopyroxene crystals with interstitial plagioclase occurs at the base—this is the PGE-bearing Merensky pegmatoid.

As part of a detailed study of the Merensky unit at Rustenburg Platinum Mines in the western lobe of the Bushveld Complex, we have determined $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (Table 1) on a suite of whole rocks and plagioclase collected from these two cyclic units and the immediate footwall to the Merensky pegmatoid. The variation of initial Sr-isotope ratios with height (Fig. 1) indicates a step-like pattern which confirms, in general, the results of Hamilton¹.

Plagioclase separates from the Footwall unit and the Merensky pegmatoid and pyroxenite have a constant initial $^{87}\text{Sr}/^{86}\text{Sr}$ which, significantly, is identical to that for rocks from the same stratigraphic interval in the eastern lobe¹. With height through the Merensky unit we find a dramatic increase in $^{87}\text{Sr}/^{86}\text{Sr}$ for both whole rocks and plagioclase. In a 60-m section through the overlying Bastard unit the initial ratios are more uniform, but not constant, between 0.70749 and 0.70803, which is within the range found by Hamilton for the main zone of the eastern lobe.

This pattern of initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio variation can help constrain processes occurring at this level during the crystallization of the Bushveld Complex. In general, two main models can be envisaged. First, the increase in $^{87}\text{Sr}/^{86}\text{Sr}$ through the Merensky cyclic unit and the high values (for mafic rocks) for main zone samples might reflect *in situ* contamination of the existing magma by its wall rocks. Alternatively, the main zone rocks might have crystallized from an influx of new magma (with

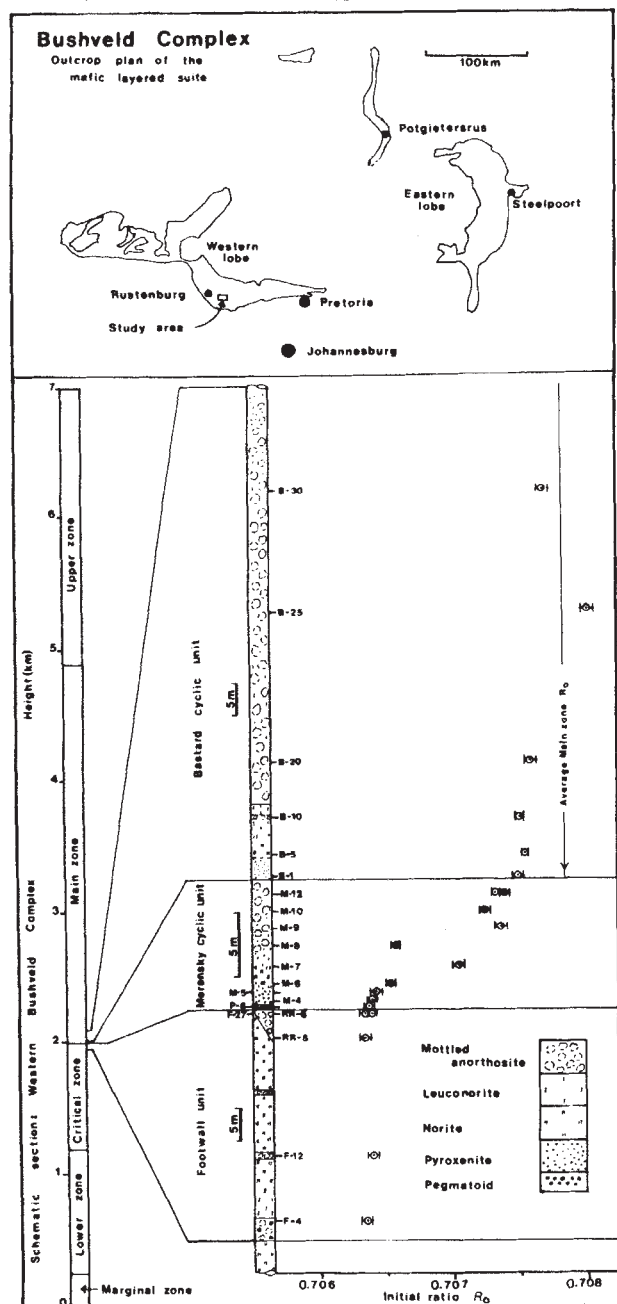


Fig. 1 Location map and stratigraphical positions for samples for which initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios have been determined. Note the differences in scale between the Merensky and Bastard and Footwall units. The average main zone initial $^{87}\text{Sr}/^{86}\text{Sr}$ (R_0) ratio is from ref. 1. ○, Plagioclase; ●, whole rocks.

$^{87}\text{Sr}/^{86}\text{Sr} > 0.7075$) into the chamber and the Sr-isotope variation in the Merensky unit reflects the interaction of this new magma, or its crystallization products, with existing material in the magma chamber.

For the case of *in situ* contamination, magma with $^{87}\text{Sr}/^{86}\text{Sr} = 0.7064$ crystallized to produce the footwall sequence. At the start of accumulation of the Merensky unit, the existing magma became contaminated with more radiogenic Sr, presumably by mixing with partial melts generated in the roof and walls of the intrusion. The increasing $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in the Merensky cyclic unit would therefore reflect progressive contamination of the magma and the increasing incorporation of more radiogenic Sr into cumulus minerals that build the cyclic unit. Contamination ceased at the level of the base of the Bastard cyclic unit and crystallization proceeded to produce rocks of the main zone, which has elevated and more uniform $^{87}\text{Sr}/^{86}\text{Sr}$ ratios.

Most of the data necessary for evaluating the feasibility of this model are unavailable, but considerations presented below suggest that *in situ* contamination is an unlikely explanation for the Sr-isotope variations. It seems unlikely that the contamination process was as short-lived as would be suggested by the thinness of the Merensky unit, and that it ceased abruptly, as indicated by the more uniform ratios in the Bastard unit. During the short contamination interval the magma must have been rapidly and efficiently mixed because rocks at this level in both the eastern and western lobes of the complex have similar Sr-isotope compositions (see Table 1 and ref. 1). If contamination occurred at the roof of the intrusion, transport of the contaminating Sr to the crystallization front at the base implies whole-magma convection. Although this possibility cannot be excluded, it seems more likely that contamination at the roof would cause self-stratification of the magma with convection cells confined to several layers⁵, thus inhibiting thorough mixing of the magma. In the Rustenburg area the Merensky unit is orders of magnitude thinner than the main zone, and, using the principle of balancing the heat budget by assuming that the amount of melting in the wall rocks is roughly equivalent to the amount of crystallization, we conclude that a very small proportion of contaminant, much less than 1%, would be mixed in with the main zone. To produce the significant elevation of $^{87}\text{Sr}/^{86}\text{Sr}$ from 0.7064 to 0.7075 the contaminant must have very high Sr and/or $^{87}\text{Sr}/^{86}\text{Sr}$. Transvaal supergroup sediments that host the complex have low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios^{1,7}. Felsites of the Rooiberg group which form the roof of the intrusion have low Sr concentrations (average 70 p.p.m.; ref. 8) although no Sr-isotope data are available. Assuming that the precontaminated magma had Sr = 250 p.p.m. (calculated from Sr-concentration of cumulus plagioclase crystals in the Footwall unit) and that the extent of contamination was 1%, then the contaminant must have $^{87}\text{Sr}/^{86}\text{Sr} > 1.0$. This reduces to > 0.78 for 5% contamination. These ratios are high for felsites with Rb/Sr ~ 2.5 that were erupted shortly before emplacement of the Bushveld Complex and whose crustal or mantle precursors had very much lower Rb/Sr ratios.

The formation of main zone rocks from one or more new influxes of gabbroic magma is favoured by several workers^{1,9-11}.

Table 1 Results of Rb/Sr analysis

Sample	$^{87}\text{Sr}/^{86}\text{Sr}^*$	Rb (p.p.m.) [†]	Sr (p.p.m.) [†]	Rb/Sr	$^{87}\text{Sr}/^{86}\text{Sr}^\ddagger$
Bastard cyclic unit					
B-30CPL§	0.70872 ± 4	4.7	398	0.0118	0.70771
B-25CPL	0.70849 ± 4	2.2	403	0.0054	0.70803
B-20CPL	0.70819 ± 4	2.7	392	0.0070	0.70760
B-10CPL	0.70787 ± 3	1.7	407	0.0041	0.70752
B-5PL	0.70806 ± 2	2.4	416	0.0059	0.70756
B-1IPL	0.70806 ± 4	2.6	385	0.0067	0.70749
Merensky cyclic unit					
M-12WR	0.70776 ± 3	1.7	403	0.0042	0.70740
M-12CPL	0.70757 ± 3	1.2	419	0.0029	0.70732
M-10WR	0.70789 ± 2	2.9	386	0.0075	0.70725
M-9CPL	0.70765 ± 4	1.4	425	0.0033	0.70737
M-8WR	0.70699 ± 2	1.9	394	0.0047	0.70659
M-7CPL	0.70731 ± 4	1.2	392	0.0030	0.70705
M-6WR	0.70696 ± 2	1.4	322	0.0044	0.70655
M-5IPL	0.79723 ± 4	4.4	469	0.0094	0.70643
M-4IPL	0.79733 ± 2	4.9	457	0.0108	0.70641
P-6IPL	0.70665 ± 2	1.7	497	0.0033	0.70637
Footwall unit					
RR-6PL	0.70661 ± 3	1.3	477	0.0027	0.70638
RR-5CPL	0.70672 ± 4	2.1	483	0.0044	0.70634
F-27CPL	0.70653 ± 5	1.1	482	0.0022	0.70634
F-12CPL	0.70663 ± 4	1.3	486	0.0027	0.70640
F-4CPL	0.70665 ± 4	1.6	479	0.0034	0.70636

* Present day $^{87}\text{Sr}/^{86}\text{Sr}$ ratio ± 2 s.e.m. NBS-987 = 0.71026 ± 2 during this study.

† Rb and Sr concentrations were determined by high precision Wd-XRF.

‡ Initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio calculated using an age of 2,100 Myr and $\lambda = 1.42 \times 10^{-11} \text{ yr}^{-1}$ for ^{87}Rb .

§ PL, plagioclase; WR, whole rock; CPL, cumulus plagioclase chadacrysts hosted by orthopyroxene; IPL, intercumulus plagioclase.

Thermal and density contrasts between existing and new liquids and the nature of intrusion of the new magma would cause convective or turbulent mixing¹²⁻¹⁵ of a presently unpredictable nature, as the exact properties of the magmas, the shape of the magma chamber, the rate of intrusion, and the position and shape of the conduits are unknown. Our detailed mineral and whole-rock composition data suggest that the new magma was compositionally similar to the existing magma, except that $^{87}\text{Sr}/^{86}\text{Sr}$ was >0.7075 and possibly >0.7086 , as available data for the main zone (Table 1; ref. 1) vary between these limits.

The pattern of Sr-isotope variation (Fig. 1) precludes a behaviour suggested by some experiments^{3,12} whereby the new magma flows to the bottom of the chamber displacing the existing magma upwards and after crystallizing a layer of crystals, mixes with it by convection. A more compelling model involves mixing of the magmas as the new influx enters the chamber. One possibility is that the deposition of the Merensky cyclic unit was concomitant with progressive influx and efficient mixing of the new magma into the existing liquid. The increasing $^{87}\text{Sr}/^{86}\text{Sr}$ ratios would therefore reflect the increasing proportion of new magma in the crystallizing mixture. The isotope data require that the influx ceased at the time the base of the Bastard unit was accumulating but the development of cyclic units and the similarity of mineral composition variations through the Merensky and Bastard units are difficult to reconcile with this restriction.

The possibility we prefer is one where the new magma entering the chamber mixes with the existing liquid and the Merensky and succeeding cyclic units form by accumulation of crystals precipitating from the blended magmas. This model would presumably result in a sharp step-like increase in $^{87}\text{Sr}/^{86}\text{Sr}$ at the base of the Merensky unit, contrary to what is observed. However, we suggest that whole-rock $^{87}\text{Sr}/^{86}\text{Sr}$ ratios in the Merensky unit were subsequently modified by infiltration of intercumulus liquid from the Footwall unit, a process described elsewhere^{3,16}. This upward migrating liquid would displace or mix with that in the Merensky unit and the proportion of Sr with the isotopic signature of the footwall would decrease with height through the overlying crystal pile. The $^{87}\text{Sr}/^{86}\text{Sr}$ of the intercumulus material would therefore vary with the proportion of Sr introduced from the footwall, and the pattern depicted in Fig. 1 could result.

Although we have not been able to demonstrate directly the mixed isotopic character of Merensky cyclic unit rocks, comparison of data for M-7CPL with M-8WR (Table 1) is suggestive. Sample M-7CPL is of plagioclase primocrysts separated from enclosing orthopyroxene oikocrysts and these have $^{87}\text{Sr}/^{86}\text{Sr} = 0.70705$, approaching values typical of the main zone. The whole-rock sample M-8 comprises plagioclase primocrysts and intercumulus plagioclase and orthopyroxene and has $^{87}\text{Sr}/^{86}\text{Sr} = 0.70659$ which is close to values typical of the Footwall unit despite its higher stratigraphical position. Three intercumulus plagioclase separates for the basal pyroxenites and the pegmatoid of the Merensky unit have $^{87}\text{Sr}/^{86}\text{Sr}$ ratios that are almost identical, within error, to the Footwall unit initial ratio. We recall that Hamilton¹ found similar ratios in pyroxenites from the lower part of the Merensky unit; this is consistent with our data because in these rocks most of the Sr is contained in the intercumulus phases.

Our data support a role for a new magma influx, magma mixing and the process of infiltration metasomatism in the genesis of the Merensky pegmatoid. At this stage the implication for the origin of the associated PGE mineralization is unclear, as isotopic variations across the PGE-mineralized UG-2 layer have yet to be documented. If our interpretation of the Sr-isotope data for the Merensky unit is valid, models for PGE mineralization in layered igneous rocks should consider these processes.

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Age constraints on the proposed Plio-Pleistocene boundary stratotype at Vrica, Italy

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Estimates¹⁻⁴ of the age of the stratotype Plio-Pleistocene boundary in Italy range from 1.65 to 2.5 Myr. We aim here to clarify this dating confusion, and we present new radiometric data on ashes from the proposed stratotype section, Vrica, Italy which indicate that the Plio-Pleistocene boundary must be less than 2 Myr old. Biostratigraphical criteria—the first appearance datums (FADs) and last appearance datums (LADs) of planktonic foraminifera and calcareous nannoplankton tied to the magnetic reversal chronology—suggest that this boundary may be nearer to 1.7 Myr. Attempts to make the boundary far older than this are without basis.

Haq *et al.*¹ have applied the calcareous nannoplankton and planktonic biochronological framework established from palaeomagnetically-dated deep sea cores to the section at Le Castella, southern Italy and estimated an age of 1.6 Myr for the Plio-Pleistocene boundary. (This age is now revised to 1.65 Myr based on the use of the newly adopted decay constants for ⁴⁰K (ref. 5).) Note that although the section at Le Castella was proposed at the Seventh INQUA Congress as a section suitable for defining the Plio-Pleistocene boundary stratotype

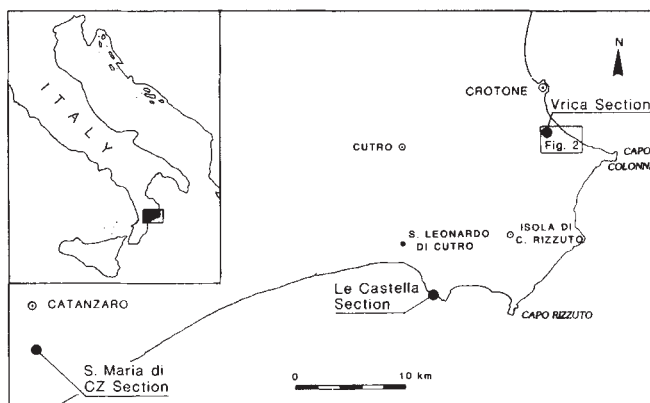


Fig. 1 Location of the Plio-Pleistocene sections at Vrica and Le Castella, and sampling localities (Calabria, Italy).

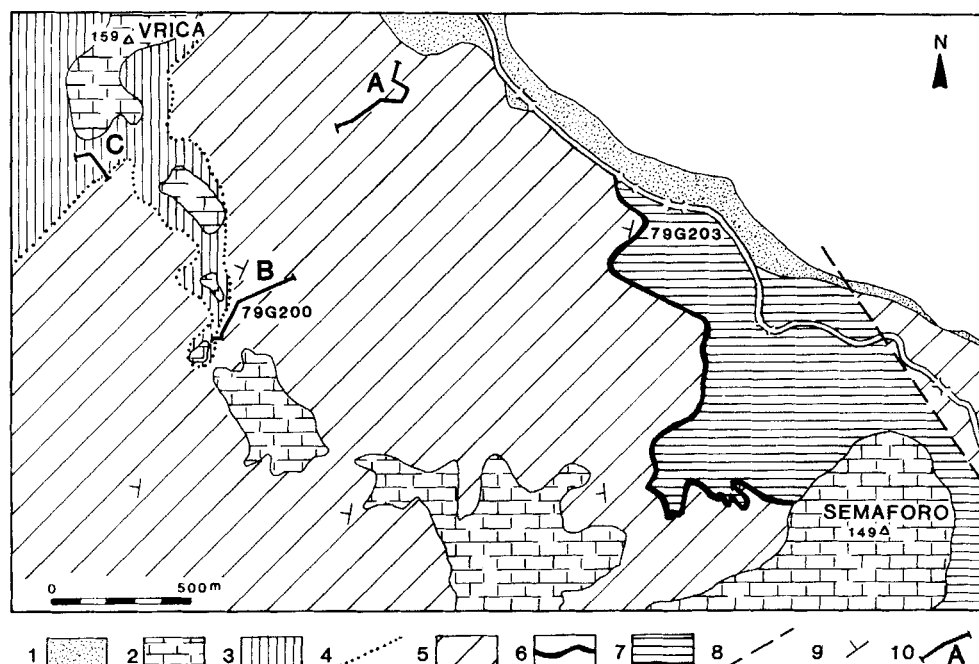


Fig. 2 Geological sketch map of the Vrica area, modified from Selli *et al.*³. 1, Beach sand and dunes; 2, calcarenites and sands of the marine terrace ('Milazzian'); 3, grey marly silty clays with sapropelic intercalations (early Pleistocene); 4, first appearance of *Hyalinae baltica*, 35 m above the *m* marker bed ash (< 1.99 Myr); 5, grey marly silty clays with sapropelic intercalations (early Pleistocene, upper and middle Pliocene; the terminology for the Pliocene follows that of Italian workers); 6, lower volcanic ash horizon (2.22 Myr); 7, grey clays (middle Pliocene); 8, faults; 9, dip; 10, intervals sampled for the reconstruction of the columnar section going from the base of unit X to the top of unit Z (see Fig. 3, after ref. 16.).

this recommendation has never been officially accepted. Moreover, Colalongo *et al.*⁶ have demonstrated the presence of a hiatus at the height of the 'marker bed' of Emiliani *et al.*⁷ (considered by many to coincide practically with the Plio-Pleistocene boundary) at the Le Castella section^{8,9}. Selli *et al.*³ proposed that a new boundary stratotype at the Vrica section (Figs 1, 2) be accepted because of the continuity of section, better exposures, a thicker interval above and below the boundary, and an equally good fossil record. They reported several age determinations on a volcanic ash, termed the *m* marker bed, and a pumice block immediately above it in the short interval where the Plio-Pleistocene boundary would fall (Fig. 3). These ages, based on the K-Ar (Savelli in ref. 3) and fission-track, F-T (Bigazzi and Bonadonna in ref. 3) methods, ranged from 2.0 to 2.2 Myr. An even older age of 2.5 Myr has been determined by Boellstorff⁴ using the F-T method on glass shards from the *m* marker bed. All of these ages, however, are far older than the age suggested by Haq *et al.*¹. Given these divergent results we collected ash samples at Vrica and other localities where volcanic tephra are associated with sediments of late Pliocene or early Pleistocene age. At Vrica we collected the *m* marker bed ash and a Pliocene ash that is 280 m lower in the section.

Collecting the Pliocene ash was comparatively easy as the ash is thick (20 cm) and its lower part is relatively coarse. This ash is characterized by the presence of hornblende, plagioclase, and zircon. We obtained concordant ages of 2.22 ± 0.03 Myr

(K-Ar, hornblende); 2.0 ± 0.16 Myr (F-T, zircon), and 2.2 ± 0.2 Myr (F-T glass) (Tables 1a and 2). However, these results are: (1) in disagreement with the ages of 3.1 and 3.4 Myr previously determined by Dymond (in Selli¹⁰) using the K-AR method on glass shards from separate samples of the same Pliocene ash bed. One of these samples comes from the same locality where we collected our sample (79G203 in Fig. 2), the other comes from the environs of St Leonardo di Cutro (ref. 10 and R. Selli, personal communication); (2) younger than the 2.5 Myr age reported by Boellstorff⁴ for the *m* marker bed ash which is 280 m higher in the section at Vrica; (3) the same as the 2.2 ± 0.2 Myr age determined by Savelli³ for glass shards from the *m* marker bed ash, though recalculation based on the data given in ref. 3 indicates an age of 2.27 Myr; and (4) almost the same age for the *m* marker bed ash of 2.07 ± 0.33 Myr determined by Bigazzi and Bonadonna on glass shards (in ref. 3). Clearly something was wrong.

It was found that the sample of ash dated by Bigazzi and Bonadonna inadvertently had two labels attached to it, one indicating the sample to be the Pliocene ash and the other the *m* marker bed ash. Instrumental neutron activation analysis (INAA) of the glass dated by Bigazzi and Bonadonna and that of the Pliocene ash we collected in 1979 indicate that they are indeed the same ash. That is, the supposed *m* marker bed ash dated by Bigazzi and Bonadonna is actually the Pliocene ash and their result of 2.07 ± 0.33 Myr agrees with our determinations.

Table 1 Analytical data for the K-Ar age determinations

DKA no.	Sample no.	Mineral	%K	Moles $^{40}\text{Ar}^*$ g $^{-1}$ ($\times 10^{-11}$)	% Rad. Ar	Age (Myr) $\pm 1\sigma$
a, Pliocene ash						
3864	79G203	Hornblende	0.805 (FL)	0.311	40.8	2.22 ± 0.05
3936	79G203	Hornblende	0.781 (ID)	0.301	32.9	2.22 ± 0.04
b, m Marker bed ash						
3861	79G201 (-80m)	Biotite (S)	7.21 (FL)	3.250	33.5	2.60 ± 0.05
3935	79G201 (+80m)	Biotite (S)	7.21 (FL)	2.882	32.1	2.30 ± 0.05
3965	79G200 (-80m)	Biotite (P)	7.03 (FL)	2.601	23.3	2.13 ± 0.05
4128	79G200 (+80m)	Biotite (P)	6.99 (ID)	2.410	12.5	1.99 ± 0.08
c, Vrica pumice						
4201		Hornblende	0.377 (ID)	0.154	7.6	2.35 ± 0.16

Decay constants for ^{40}K (ref. 5): $^{40}\text{K}/\text{K} = 1.167 \times 10^{-2}$ atom %; $\lambda_\beta = 4.962 \times 10^{-10} \text{ yr}^{-1}$; $\lambda_e + e' = 0.581 \times 10^{-10} \text{ yr}^{-1}$. ID, isotope dilution; FL, flame photometry.

Table 2 Analytical data for the F-T age determination

Sample no.	Mineral	Correction method ¹⁷	D^S/D^I or Temperature	$\rho_s \times 10^5$ tracks cm ⁻²	$\rho_i \times 10^{15}$ tracks cm ⁻²	$\Phi \times 10^{15}$ n cm ⁻²	App. age (Myr)	Corr. age (Myr)	$\pm 1\sigma$ (Myr)	Grains	$r, \bar{s}$
79G203	Zircon	—	—	3.21 (95)	94.8 (1404)	1.01	2.0	—	0.16	12	0.98 (r)
79G203	Glass	size	0.83	0.0281 (316)	0.235 (1046)	0.190	1.37	1.9	0.2	200/130	3.8% (s)
79G203	Glass	plateau	200 °C	0.0272 (307)	0.144 (560)	0.190	—	2.2	0.2	230/100	4.8% (s)

D^S/D^I , size ratio of spontaneous to induced tracks, shown for the size corrected age only. Temperature, heating temperature for the plateau age. ρ_s , ρ_i , Spontaneous and induced track densities respectively. (), Number of tracks counted. Φ , Neutron fluence, referred to the standard SRM 962 of the NBS. App. age, apparent age before any correction. Corr. age, corrected age. Grains, the number of glass grains used for counting the spontaneous and induced tracks respectively are shown; for zircon the total number of grains counted using the external detector method. $\bar{s}$, Experimental standard deviation, in per cent, of the induced track counting; r , correlation coefficient. Decay constant for the spontaneous fission of $^{238}\text{U} = 7.03 \times 10^{-17} \text{ yr}^{-1}$; although the Denver and Pisa F-T groups routinely use slightly different values for the decay constant the same value is used here for consistency. The glass irradiations were performed in the Pavia University (Italy) reactor and the zircons were irradiated at the USGS reactor, Denver, Colorado.

J. Dymond (personal communication) revealed that unpublished $^{40}\text{Ar}/^{39}\text{Ar}$ stepwise heating data for the sample of Pliocene ash at St Leonardo di Cutro indicates that the glass shards contain excess radiogenic argon. We believe that this is also true of the other ash sample from Capo Colonne.

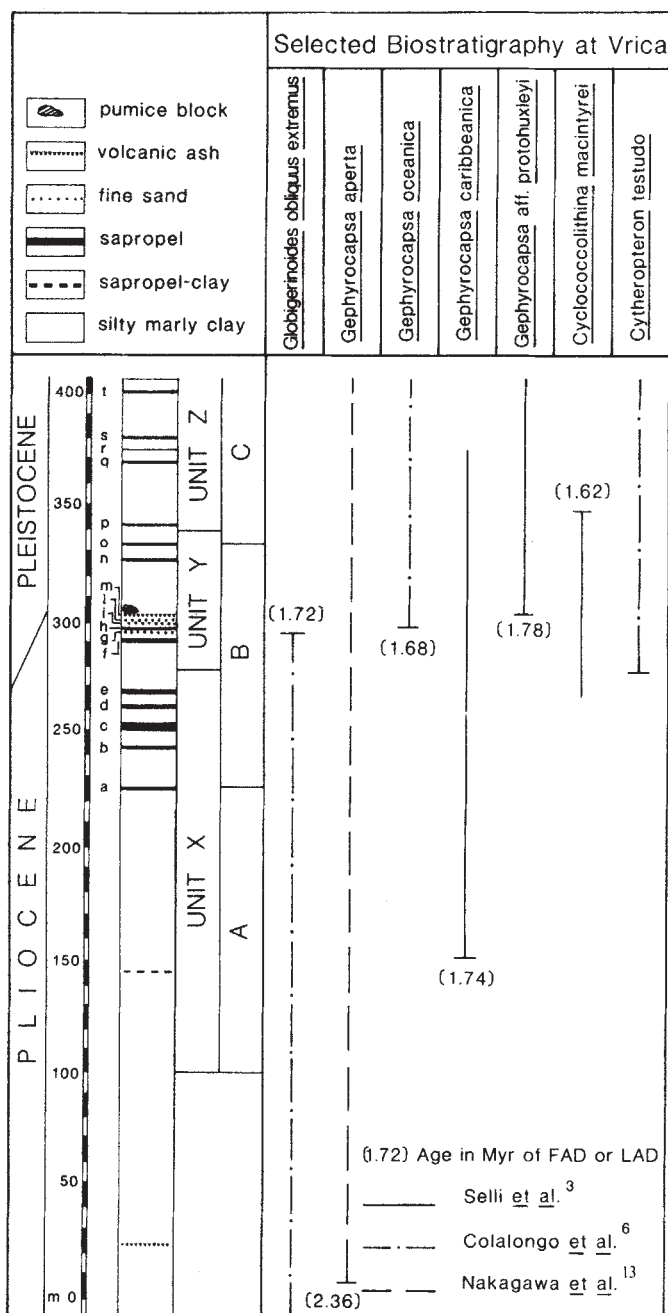
With respect to Savelli's determination of 2.27 Myr (revised) for the *m* marker bed ash we can only stress that K-Ar ages based on total fusion of glass shards are to be viewed with caution¹¹. Without independent criteria such as Dymond's $^{40}\text{Ar}/^{39}\text{Ar}$ stepwise heating approach or dating of a more reliable uncontaminated mineral phase of the ash, there is no way to appraise the results.

We think that Boellstorff probably dated the Pliocene ash but we have not been able to verify this point. However, his age of 2.5 Myr, whether for the Pliocene ash or the *m* marker bed ash, is inconsistently old compared with our radiometric data and current biostratigraphic data. The calcareous nannoplankton *Gephyrocapsa aperta* appears 17 m below the Pliocene ash at Vrica¹³. In deep sea cores *G. aperta* makes its first appearance at 2.36 Myr (ref. 2) but in the Mediterranean its appearance could be younger. With these constraints Boellstorff's result is too old for the Pliocene ash and particularly the *m* marker bed ash.

Collection of the *m* marker bed ash proved difficult because it is at most 2–3 thick and is quite lenticular. Two samples of the ash were collected. A primary sample of about 1 kg was collected very carefully to avoid any extraneous material. The exterior surface of each solid fragment of ash was removed with a knife to avoid possible contamination. A much larger secondary sample was collected with less caution. This ash is characterized by the presence of biotite and plagioclase. Unfortunately the absence of zircon prevented dating by the F-T method as the glass shards from this particular ash are not suited to dating by this method.

Our efforts to date the *m* marker bed ash have been only partially successful. Biotite from the secondary sample yielded an age of 2.6 Myr, older than that of the underlying Pliocene ash (see Table 1b). We concluded that the biotite sample is contaminated and separated the secondary and primary samples into two size fractions, +80 mesh and -80+100 mesh. Contamination is probably greater in the finer fraction and that fraction should yield the older age. The results in Table 1b indicate that this is so. The coarser fraction of the primary sample yielded the youngest age: 1.99 Myr.

Electron microprobe analysis for potassium, iron, magnesium, titanium, and calcium were performed on each of the four biotite separates to determine, if possible, the level of contamination. Two distinct classes of detrital biotite are evident. These are characterized by (1) higher Fe, slightly higher K, and lower Mg, (2) lower Fe, slightly lower K, and higher Mg contents than the normal population of primary biotite. Several grains which showed lowered levels for all of the elements examined were excluded from the tabulation as it was



thought that they probably represented leached biotite grains. The detrital grains can be listed as follows:

Primary (+80 mesh)	2 grains out of 102
(-80 + 100 mesh)	6 grains out of 127
Secondary (+80 mesh)	1 grain out of 110
(-80 + 100 mesh)	6 grains out of 132

The limited number of grains examined may not give a realistic appraisal of the true level of contamination but these samples are definitely contaminated.

In retrospect, then, the age of the *m* marker bed and the proposed Plio-Pleistocene boundary-stratotype which should be placed close to this level should be younger than 1.99 ± 0.08 Myr.

Savelli and Mezzetti¹², Selli *et al.*³, and Colalongo *et al.*⁶ have repeatedly stressed the importance of the presence of a block of 'perfectly preserved pumice' immediately above the *m* marker bed ash at Vrica. This statement needs to be clarified.

First, one might infer from these reports that the pumice was actually collected at Vrica. However, the pumice block was collected from a quarry west of the city of Crotone some 4 km north of the Vrica section. One of us (G.P.), however, is convinced that the level at which the pumice block was found can be correlated to a level located ~1 m above the *m* marker bed at the Vrica section. Second, there is a large dissimilarity in the mineralogy and chemistry of the pumice and the *m* marker bed ash. The pumice contains phenocrysts of hornblende and plagioclase while the ash contains biotite and plagioclase. INAA of the glass fraction indicates that the pumice block is very similar to but not an exact equivalent of the Pliocene ash.

Approximately 250 g of the pumice was sent to Denver and sufficient hornblende for a single analysis was recovered. The K-Ar age of 2.35 ± 0.16 Myr (Table 1c) obtained was interpreted as indicating that the pumice block has been reworked from an older pumice layer and thus has no relevance to the age of the *m* marker bed.

The question really is just how much younger than 1.99 ± 0.08 Myr might the actual age of the *m* marker bed be? Selli *et al.*³ used the sparsely available radiometric data and drilling efforts near Semaforo (Fig. 2) to propose rates of sedimentation of 35–48 cm kyr⁻¹ for the 1,250 m between the top of the Messinian which they assumed to be 6 or 7 Myr old and the Pliocene ash previously dated at 3.4 Myr. If we use the more commonly accepted age of 5 Myr for the Miocene-Pliocene boundary and the 2.2 Myr age for the Pliocene ash we get an average rate of 45 cm kyr⁻¹. This rate is still in agreement with Selli *et al.*³ because of the change in age of ~1 Myr at each end of the stratigraphical section. Assuming continuous sedimentation and applying this rate to the 280 m between the Pliocene ash and the *m* marker bed gives an age of ~1.6 Myr for the *m* marker bed. However, average rates of sedimentation must be used with caution.

Ultimately the resolution of the problem of the age of the *m* marker bed and, of course, the age of the Plio-Pleistocene boundary may rest on biostratigraphical and palaeomagnetic criteria. Nakagawa *et al.*¹³ have presented a preliminary version of the magnetostratigraphy of the Vrica section, but later circulated results and interpretations quite different and conflicting: therefore the treatment of this topic is at present impractical. Thus we give here only a brief treatment of the relevant biostratigraphy at Vrica.

Berggren *et al.*² have summarized the numerous first appearance datums (FADs) and last appearance datums (LADs) of certain planktonic foraminifera and calcareous nannoplankton which have been recognized in deep sea cores and related to the magnetic reversal time scale. In using FADs and LADs one must keep in mind that the first appearance datum at any specific geographical locality may not coincide in time and may be younger than the first evolutionary appearance datum just as the last appearance datum may simply represent the disappearance of a particular species from a certain locality or region

and so may be older than the true world-wide extinction of that species. Among the FADs and LADs mentioned by Berggren *et al.*² and by Haq *et al.*¹ the following have been recognized at Vrica (see Fig. 3 for the distribution within the section).

Planktonic foraminifera: (1) LAD of *Globigerinoides obliquus* occurs in deep sea cores at the top of the Olduvai normal polarity event with an interpolated age of 1.72 Myr (ref. 2). At Vrica *G. obliquus* is recognized at the subspecific level as *G. obliquus extremus* but this is in the same concept of *G. obliquus* recognized by Haq *et al.*¹, mentioned by Berggren *et al.*² and confirmed by Berggren (personal communication). **Calcareous nannoplankton:** (1) LAD of *Cyclococcolithina macintyreii* occurs in deep sea cores above the Olduvai normal polarity event and has an interpolated age of 1.62 Myr (ref. 2, p. 285). (2) FAD of *Gephyrocapsa oceanica* in deep sea cores occurs just above the top of the Olduvai normal polarity event and has an interpolated age of 1.68 Myr (ref. 2, p. 284). (3) FAD of *Gephyrocapsa caribbeanica* in deep sea cores occurs just below the top of the Olduvai normal polarity event and has an interpolated age of 1.74 Myr (ref. 2, p. 285). The utility of the FAD of this species may be reduced because of conflicting concepts of it held by various investigators (ref. 14, p. 19). If the FAD of *G. caribbeanica* at Vrica section really occurs 73 m below sapropel layer *a* as indicated by Selli *et al.*³ (Fig. 3), then we are faced with a troublesome fact. The FADs of *G. oceanica* and *G. caribbeanica* are separated by some 140 m of sediment. according to Berggren *et al.*² this interval would represent ~60,000 yr giving an average rate of sedimentation of ~230 cm kyr⁻¹, an inordinately high rate for this part of the section. (4) FAD of *Gephyrocapsa protohuxleyi* in deep sea cores is within the top of the Olduvai normal polarity event but occurs slightly before the FAD of *G. caribbeanica* (ref. 1, Fig. 5). If we adopt for the Olduvai the age limits proposed by Berggren *et al.*² (1.72–1.88 Myr), the FAD of *G. protohuxleyi* has an interpolated age of 1.78 Myr. At Vrica *G. aff. protohuxleyi* has been recognized and occurs ~10 m above the FAD of *G. oceanica*. (5) FAD of *Gephyrocapsa aperta* occurs in deep sea cores below the Olduvai normal polarity event and has an interpolated age of 2.36 Myr (ref. 2, p. 284). At Vrica *G. aperta* makes its first appearance 17 m below the Pliocene ash whose age is 2.22 Myr.

Figure 3 shows the age assignments for the various FADs and LADs in parentheses. Despite the qualifications cited above an age of ~1.7 Myr for the *m* marker bed would be consistent with the biostratigraphical data.

According to the recent resolution of the joint meeting of the INQUA Subcommittee 1-a (= ICS Working Group N/Q Boundary) and the IGCP Project 41 made during the International Geological Congress in Paris (July 1980), "the N/Q boundary should be placed in the Vrica section taking into account the FAD of the early cold guest *Cytheropteron testudo* (whatever its palaeoclimatic significance could be), which occurs in Vrica 10 m above Sapropel *e*. [Colalongo *et al.*^{6,9}, and Colalongo and Sartoni¹⁵, have demonstrated that in the Vrica section the FAD of *C. testudo* is accompanied by palaeontological events (FADs and LADs of planktonic foraminifera and calcareous nannofossils) that are useful for long-distance correlations.] On the other hand, there are alternative possibilities for selecting a level as the N/Q boundary stratotype in the Vrica section, within the stratigraphical interval between level *e* and the volcanic ash level *m*, which are closely related with the FAD of *C. testudo*, within a reasonably short time range."

Taking this resolution into account, it is apparent that the proposed Plio-Pleistocene Boundary stratotype in the Vrica section will fall close to 1.7 Myr and that all other published estimates suggesting an age of 2 Myr or more are inappropriate.

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Brown adipose tissue thermogenesis is 'suppressed' during lactation in mice

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Brown adipose tissue is considered to be the main site of thermoregulatory non-shivering thermogenesis in the newborn of many mammalian species, in arousing hibernators, and in adult cold-adapted rats and mice^{1–4}. Several recent studies have now suggested that the tissue may also be important in the regulation of energy balance. Evidence has been obtained indicating that the ability of genetically obese (*ob/ob* and *db/db*) mice to become obese on a normal energy intake is mainly due to reduced brown adipose tissue thermogenesis^{5–7}, and a reduction in the activity of the tissue has also been observed in rats made obese by lesions of the ventromedial hypothalamus⁸. In contrast, increases in thermogenesis in brown adipose tissue have been implicated in the immediate thermic effect of food⁹ and in cancer cachexia¹⁰, and a substantial augmentation of the activity of the tissue has been demonstrated in rats and mice exhibiting regulatory diet-induced thermogenesis as a response to chronic voluntary over-feeding^{11–14}. Whether or not brown adipose tissue thermogenesis changes in other, more physiological, states where energy flux and metabolic efficiency are altered (independently of environmental temperature) has not been established. We now report that the thermogenic activity of brown adipose tissue is suppressed in mice during the 'physiological hyperphagia' of lactation, and show that this probably results in a substantial reduction in the energy requirement for maintenance of the lactating animal.

The animals used in this study were mice of the 'Aston' variety¹⁵, aged between 2½ and 4 months. Pregnant and lactating animals were taken during their first or second litter, and each litter contained between 6 and 10 pups. Virgin females, housed in pairs, were used as controls. The temperature of the animal house was maintained at 21 ± 2 °C, and the mice were subject to a 12 h light/12 h dark cycle (light period from 07.00 h). All mice were given free access to both water and a low-fat commercial breeding diet (Spillers–Spratts Rodent Breeding Diet 1).

In the first experiments the weight, total protein content and cytochrome oxidase activity (E.C.1.9.3.1) of brown adipose tissue from the readily accessible interscapular site were determined for virgin mice and for mice at mid-lactation (days 9–12 post partum). A comparison was also made with mice during late pregnancy (see Table 1). The total amount of interscapular brown adipose tissue was greater in both the lactating and pregnant animals than in the virgin mice. This increase is probably due to increased lipid deposition, as the total protein content of the tissue was lower in both the pregnant and lactating animals. The cytochrome oxidase activity in brown

Table 1 Weight, protein content and cytochrome oxidase activity of interscapular brown adipose tissue from virgin and lactating mice

	Virgin	Pregnant	Lactating
Tissue weight (mg)	68.5 ± 3.8 (17)	102.6 ± 8.8‡ (6)	93.7 ± 3.5‡ (12)
Total protein content (mg)	11.7 ± 0.5 (17)	9.6 ± 0.4* (6)	8.2 ± 0.4‡ (12)
Total cytochrome oxidase activity (µmol cytochrome c oxidized per min)	17.5 ± 0.8 (15)	13.2 ± 0.6† (6)	8.8 ± 1.1‡ (10)

Mice were killed by cervical dislocation and the interscapular brown adipose tissue rapidly removed, and trimmed free of other tissues. The cytochrome oxidase activity of brown adipose tissue was measured spectrophotometrically²⁸ and the total protein content assayed by a modified Lowry method²⁹ using BSA as a standard. The results are given as mean ± s.e.m., with the numbers of animals shown in parentheses.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$, compared with the virgin animals (Student's *t*-test).

adipose tissue of the lactating mice was reduced to half the level in the virgin animals, indicating that during lactation there is a substantial decrease in the overall oxidative capacity of the tissue. The cytochrome oxidase activity was also reduced in late pregnancy, to a value between that of the virgin and lactating animals.

The principal mechanism for thermogenesis in brown adipose tissue is via a proton conductance pathway across the inner mitochondrial membrane, which results in the uncoupling of respiration from ATP synthesis¹⁶. The proton conductance pathway can be blocked by purine nucleotides, such as GDP, which bind to a specific 'uncoupling' protein of molecular weight 32,000 (refs 17, 18). The activity of the proton conductance pathway is generally assessed from the extent to which radioactively labelled purine nucleotides bind to brown adipose tissue mitochondria^{19–22}. Table 2 shows the results of a GDP-binding study of virgin, pregnant and lactating mice. Compared with the virgin mice, GDP binding was much lower in both early (days 1–3 post partum) and mid-lactation; it was also low during late pregnancy. Binding was lowest at mid-lactation and was not significantly increased 24 h after the pups had been abruptly weaned. These results suggest that the activity of the thermogenic proton conductance pathway is substantially reduced during pregnancy and lactation in mice.

The full extent of the reduction in brown adipose tissue thermogenesis in lactating mice was assessed by reference to the level of GDP binding in virgin animals acclimated for 3 weeks at an environmental temperature (33 °C) where there is

Table 2 GDP binding to brown adipose tissue mitochondria from virgin and lactating mice

	GDP bound (pmol per mg mitochondrial protein)
Virgin	249.4 ± 24.7 (10)
Pregnant	88.0 ± 7.4 (7)*
Lactating (early)	93.3 ± 14.6 (6)*
Lactating (mid)	56.2 ± 5.5 (8)*
Abruptly weaned at mid-lactation	65.5 ± 5.0 (6)*
Virgin, adapted at 33 °C	55.1 ± 5.0 (9)*

Mice were killed by cervical dislocation and brown adipose tissue rapidly removed from the interscapular, subscapular and dorso-cervical sites. Mitochondria were then prepared as described by Cannon and Lindberg³⁰. GDP binding was measured by incubating the mitochondria with 10 µM [³H]-GDP at 20 °C, pH 7.1, for 7 min, as described previously^{7,14}. [³H]-GDP and [¹⁴C]-sucrose were obtained from Amersham.) The results are given as mean ± s.e.m., with the number of observations shown in parentheses. For each assay, mitochondria were obtained from 1–2 mice.

* $P < 0.001$, compared with the virgin animals (Student's *t*-test).

Table 3 Respiration rates in brown adipose tissue mitochondria from virgin and lactating mice

Additions	Mitochondrial respiration (nmol O ₂ per min per mg mitochondrial protein)	
	Virgin	Lactating (mid)
–	110.7 ± 18.9	53.3 ± 9.0*
BSA (2.5 mg ml ⁻¹)	308.2 ± 15.3	188.2 ± 13.4†
GDP (1 mM)	131.1 ± 10.8	149.4 ± 10.3(NS)
Change in rate with GDP	–177.2 ± 18.6	–38.8 ± 8.7†

Brown adipose tissue mitochondria were prepared as described in Table 2 legend. Respiration was measured at 25 °C in a Yellow Springs YSI model 53 oxygen electrode, using medium (pH 7.1) containing 100 mM sucrose, 10 mM glycylglycine and 5 µM rotenone, with 10 mM α -glycerophosphate as substrate²⁴. The results are mean $\pm$ s.e.m. for 6 groups of virgin and 6 groups of lactating mice (each group consisted of mitochondria obtained from 2 mice).

* $P < 0.05$; † $P < 0.001$, compared with the virgin animals (Student's *t*-test). NS, not significant ($P > 0.05$).

no requirement for non-shivering thermogenesis²³. The results obtained with mice in these conditions (Table 2) were almost identical to those observed at mid-lactation. A Scatchard analysis of binding data showed that the reduced GDP binding in lactating mice was due to a reduction in the number of binding sites, and not to any change in affinity; the apparent dissociation constant (K_D) was approximately 3 µM at pH 7.1 for both virgin and lactating animals.

The GDP-binding assay is a physicochemical index of the proton conductance pathway. To obtain a more direct physiological measure of the thermogenic activity of brown adipose tissue mitochondria during lactation, mitochondrial respiration was determined (with α -glycerophosphate as substrate) in the presence and absence of exogenous GDP. The results in Table 3 show that in the presence of bovine serum albumin (BSA), which removes the inhibitory effects of endogenous fatty acids on the activity of α -glycerophosphate dehydrogenase^{24,25}, the mitochondrial respiration rate was significantly higher in virgin than in lactating mice. Following the addition of GDP, to recouple the mitochondria and restore respiratory control, the respiration rate fell much more in the virgin than in the lactating mice, and there was no longer any significant difference between the two groups. The difference between the respiration rates before and after adding GDP was almost 5 times higher for the virgin than for the lactating mice; thus, brown adipose tissue mitochondria from lactating mice seem to be much more fully 'coupled' than those from virgin animals.

The results presented here clearly demonstrate that thermogenesis in brown adipose tissue is suppressed in lactating mice, and the suppression is such that the thermogenic activity is similar to that of mice adapted to thermoneutral conditions. Alterations in the metabolism of brown adipose tissue in lactation have been observed previously by Agius and Williamson^{26,27} in studies on lipogenesis. Not only is thermogenesis for thermoregulatory purposes inhibited in brown adipose tissue during lactation, but the increased food intake of the lactating animal does not result in a dietary-induced stimulation of the tissue—in contrast to the response invoked during overfeeding in normal, non-lactating, rats and mice^{11–14}. This is, of course, a rational response as substrates which would otherwise be dissipated in brown adipose tissue as heat are available for the synthesis of milk^{26,27}.

The inhibition of brown adipose tissue thermogenesis would be expected to have a significant energy sparing effect on the lactating animal. To obtain an estimate of the possible (maximum) quantitative importance of such an effect, we have measured the energy intake of virgin mice, aged 3 months, adapted at 21 °C and at 33 °C (thermoneutrality); the difference should reflect the normal energy flux through brown adipose tissue and those tissues (for example, heart) which have a supportive role in thermogenesis^{3,4,6,13}. At 21 °C the gross

energy intake of the virgin mice was 76 kJ per day, while at 33 °C it was 45 kJ per day, giving a figure of 31 kJ per day for the energy expenditure on thermogenesis at 21 °C. Thus, if *in vitro* experiments on mitochondrial respiration and GDP binding accurately reflect the activity of brown adipose tissue *in vivo*, the suppression of brown adipose tissue thermogenesis in the lactating mouse could conserve as much as 31 kJ per day, and this is equivalent to 40% of the maintenance energy requirement of the normal, non-lactating, animal.

The most likely explanation for the results obtained here is either that during pregnancy and lactation there is a distinct physiological adaptation for energy conservation (mediated by prolactin?), or that the increased metabolic heat production which results from fetal growth and milk synthesis is sufficient to obviate the need for any specific thermoregulatory heat production. Whichever explanation is correct, the inhibition of brown adipose tissue thermogenesis would be expected to have a significant effect on the overall energy expenditure and energy requirement of the lactating mouse. Whether there are implications from the observations presented here for other (larger) species, including man, is not clear. In conclusion, our results, together with a previous study on lipogenesis following intra-gastric feeding²⁶, indicate that the lactating animal may be a valuable model in which to investigate the regulation of thermogenesis in brown adipose tissue.

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Relationship of β -adrenoreceptor density to fitness in athletes

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Physical training in man results in a decreased heart rate at rest^{1,2}, associated with an apparent decrease in the activity of the sympathetic nervous system^{1,3}. One possible mechanism for this would be alterations in β -adrenoreceptor density with training. Exercise produces large increases in plasma catecholamine concentrations^{4,5}. Moreover, lymphocyte β -adrenoreceptor density in man is inversely correlated with plasma and urinary noradrenaline concentrations and is positively correlated with cardiac sensitivity to isoprenaline⁶. Thus,

alterations in β -adrenoreceptor density in the cardiovascular system might be reflected in changes in lymphocyte β -adrenoreceptor density. We have now measured lymphocyte β -adrenoreceptor density in a group of athletes of varying degrees of fitness before and after they embarked on an intensive training programme. Our results show clear correlations between degree of physical fitness and receptor density and we conclude that decreases in sympathetic nervous system responsiveness following physical training are related to decreases in β -adrenoreceptor density.

Observations were made on 16 male swimmers aged 14–24 yr. The subjects were of varying fitness and were about to embark on a 2-month intensive training programme. A venous blood sample (30 ml) was obtained from each subject at 10.30 a.m. Lymphocytes were isolated using the method of Boyum⁷ and β -adrenoreceptor density was measured by a radioligand binding method similar to that of Williams *et al.*⁸. At 11 a.m. on the same morning, each subject underwent sub-maximal exercise on a Monarch bicycle ergometer and fitness levels were then assessed using predicted aerobic capacity (maximal oxygen intake, max VO_2) from the Astrand–Rhyming nomogram⁹. Results for max VO_2 and adrenoreceptor densities were only correlated at the completion of the study. No subject was receiving any drugs.

Pre-training max VO_2 was 24–61 ml per kg per min (mean, 49.6 ± 2.3 s.e.m.) and pre-training β -adrenoreceptor density varied over 1,538–385 receptors per lymphocyte (mean,

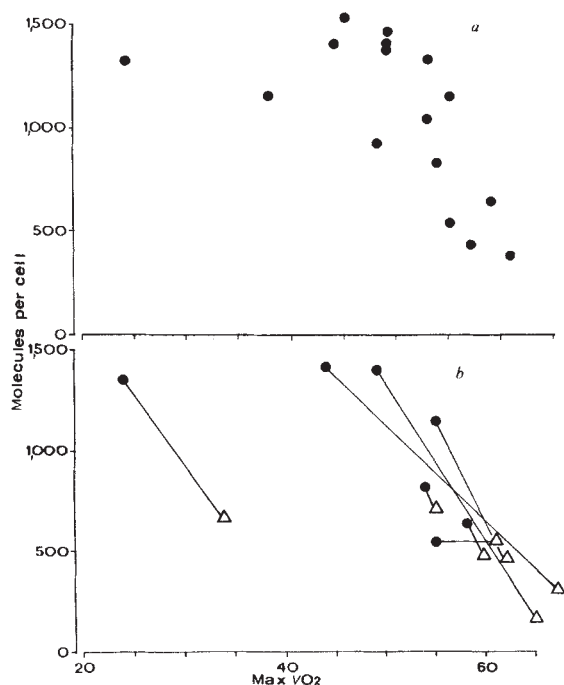


Fig. 1 *a*, Relationship between maximal oxygen intake (max VO_2 in ml per kg per min) and lymphocyte receptor density (as molecules of dihydroalprenolol specifically bound per cell), in 16 swimmers of varying fitness before embarking on a training programme. Lymphocyte β -receptor density was estimated by a method similar to that of Williams *et al.*⁸. Lymphocyte membranes (0.1 ml) equivalent to 2×10^6 cells were incubated with ^3H -dihydroalprenolol (102 Ci mmol^{-1} Amersham) at concentrations of 0.5–5.5 nM. The final volume was 0.2 ml. After incubation for 15 min at 37 °C the reaction was terminated by the addition of 2 ml ice-cold buffer (50 mM Tris, 2 mM MgCl_2 , pH 7.6). Membranes were separated by immediate filtration through Whatman GFC filters which were then washed with three 2-ml volumes of buffer. Nonspecific binding was estimated in the presence of 10^{-5} M alprenolol. Numbers of specific dihydroalprenolol binding sites were calculated using Scatchard plots. *b*, Maximal oxygen uptake (ml per kg per min) plotted against lymphocyte receptor density (as molecules of dihydroalprenolol specifically bound per cell) in seven swimmers before (●) and after (△) a 2-month training programme.

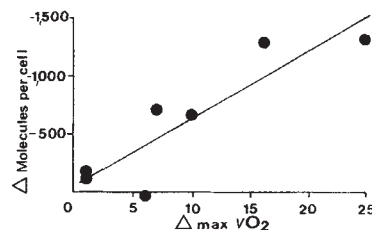


Fig. 2 Relationship between change in receptor density (as decrease in the number of molecules of dihydroalprenolol specifically bound per cell) and change in maximum oxygen intake (as increase in max VO_2 , ml per kg per min) in the seven swimmers who underwent a 2-month training programme. The line of best fit, determined by linear regression analysis, is shown (correlation coefficient = -0.89 , $P < 0.01$).

$1,062 \pm 97$ s.e.m.) (Fig. 1*a*). There was a complex inverse relationship between max VO_2 and β -adrenoreceptor density (Spearman's $\rho = 0.75$, $P < 0.001$). At low to average levels of fitness, β -adrenoreceptor density was in the region of 1,000–1,500 sites per cell, in good agreement with values previously reported by us in young people of average physical fitness¹⁰. At higher levels of fitness, however, represented by max VO_2 values greater than 45–50 ml per kg per min, receptor density decreased sharply.

Seven subjects completed their training schedule satisfactorily and were reassessed. Figure 1*b* plots pre- and post-training values for max VO_2 and receptor density for each of these subjects. Training increased max VO_2 in every case, the mean pre-training value being 48.7 ± 4.5 ml per kg compared with a mean post-training value of 58.1 ± 4.1 ml per kg ($P < 0.05$, Student's *t*-test for paired data). After training, lymphocyte β -adrenoreceptor density in these seven subjects decreased from a mean of $1,074 \pm 152$ receptors per cell to a mean of 458 ± 78 ($P < 0.05$) and in all but one subject, receptor density decreased. The single exception showed an insignificant change in receptor density from 547 to 554 sites per cell. Those subjects showing the greatest increase in fitness also tended to show the greatest fall in lymphocyte β -adrenoreceptor density and there was a significant linear correlation between change in receptor density and change in max VO_2 (Fig. 2).

Our results show a clear, inverse relationship between physical fitness and lymphocyte β -adrenoreceptor density. Physical training can further reduce β -adrenoreceptor density and this reduction is proportional to changes in fitness. The most likely explanation involves changes in concentrations of circulating catecholamines, although effects of other exercise-related metabolic processes such as metabolic acidosis cannot be excluded. Exercise results in large increases in plasma catecholamine concentrations. These increases are slightly lower in trained subjects but are still substantial⁵. High concentrations of sympathomimetic amines in man, whether from exogenous or endogenous sources, result in a decreased responsiveness of lymphocytes to stimulation by these agents¹¹, associated with decreases in receptor density^{12,13}. The reductions in receptor density at higher levels of physical fitness seen in the present study probably result from regular exposure to high concentrations of catecholamines. Fraser and colleagues⁶ recently found evidence that these reductions could result in decreased sympathetic nervous system responsiveness and showed that low lymphocyte β -adrenoreceptor densities were associated with low cardiac sensitivity to isoprenaline.

We suggest that the decreased sympathetic nervous system responsiveness seen in athletes is due to a decreased β -adrenoreceptor density resulting from chronic exposure to high concentrations of catecholamines. Teleologically, this could represent a protective mechanism against chronic sympathetic nervous system stimulation. Physical fitness should be taken into account in studies involving measuring β -adrenoreceptor density or responsiveness.

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Visual pigments in the four-eyed fish, *Anableps anableps*

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The retinæ of terrestrial and marine vertebrates generally contain the visual pigment, rhodopsin, whereas those of freshwater vertebrates contain porphyropsin^{1,2}. The latter pigment absorbs light at longer wavelengths than rhodopsin and therefore has increased red sensitivity, which is possibly beneficial to animals living in water containing suspended particles since these may cause image degradation due to light scattering at short wavelengths. Many fish and amphibians have both types of pigment, and in some fish, the proportion of rhodopsin to porphyropsin is greater in the dorsal half of the retina³. In the bullfrog, *Rana catesbeiana*, the ventral retina contains only rhodopsin, whereas the dorsal retina has appreciable amounts of porphyropsin⁴. This may be attributable to the fact that the bullfrog often has only its eyes and nose protruding above the water surface, thus the dorsal part of the retina is used for aquatic vision and the ventral part for aerial vision. The 'four-eyed' fish, *Anableps*, is able to see simultaneously in air and water; it normally swims so that the eye is bisected by the water meniscus. Each eye has a single retina, but the dorsal part receives images from the water, and the ventral region receives aerial images^{5,6}. Thus it has been suggested that the dorsal region should show more red dominance⁷, with porphyropsins being restricted to this area. We have measured the absorbance spectra of the photoreceptors, and report here that the same visual pigments are present in the dorsal and ventral regions of *Anableps* retina.

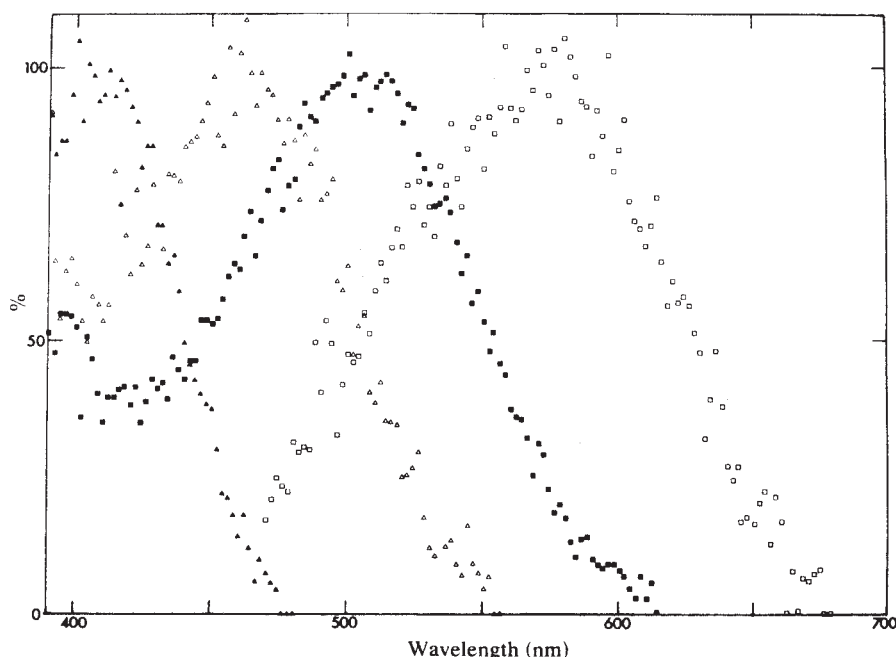
A visual pigment molecule consists of a protein moiety, opsin, and a chromophore, based on either vitamin A₁ or A₂. Rhodopsins, generally found in the retinæ of terrestrial and marine vertebrates, are based on vitamin A₁, whereas porphyropsins, found in freshwater vertebrates, are based on vitamin A₂. Using the same opsin, a vitamin A₂ pigment absorbs light at longer wavelengths than a vitamin A₁ pigment, hence porphyropsin has increased red sensitivity compared with rhodopsin.

Live specimens of *Anableps anableps* (obtained from a tropical fish supplier in London) were maintained in brackish water (specific gravity 1.005, pH 7–8, temperature 22–24 °C). The fish were dark-adapted overnight before an experiment. All procedures were then carried out under dim red light (Kodak safelight no. 2).

The fish were decapitated, and the eyes removed and stored in the dark at 4 °C until required. Each eye was cut into dorsal and ventral sections with a razor blade, using as a guide the horizontal corneal pigment band which marks externally the division between the two halves of the eye. The retina was removed from each section and placed in freshwater teleost Ringer's solution. A small piece of retina (~1 mm²) was cut from one of the retinal halves and prepared using a method similar to that described elsewhere⁸. Briefly, the tissue was cut into fragments with razor blades and squashed between two coverslips, which were sealed with paraffin wax to avoid evaporation. Microspectrophotometric measurements were made using a modified Leibman microspectrophotometer^{9,10} under computer control, as described elsewhere¹¹. The microspectrophotometer was programmed to step from 750 nm to 390 nm in 2-nm steps, taking in data at even-numbered wavelengths, and to return, taking in data at odd-numbered wavelengths.

In both regions of the retina, four distinct cell types were identified: rods, single cones and two different classes of double cone. The rods contained a visual pigment having a wavelength of maximum absorbance ($\lambda_{\max}$) 506 nm (Fig. 1), which is in

Fig. 1 Mean absorbance spectra for four classes of photoreceptor from dorsal and ventral retinal samples for three individual fish. The maximum absorbance ($\lambda_{\max}$) values of the four classes are: single cone, 409 nm ($n = 13$); rod, 506 nm ($n = 9$); middle-wave-sensitive member of double cone, 463 nm ($n = 4$); long-wave-sensitive member of double cone, 576 nm ($n = 6$). Each point represents the average of values obtained at two adjacent wavelengths, one value recorded in the descending scan, the other in the ascending scan. The mean spectra have been normalized to a maximum of 100%. For each absorbance spectrum, the approximate wavelength of peak absorbance was estimated and each of 25 individual values on either side of the provisional peak was referred to the appropriate nomogram to estimate the wavenumber of peak sensitivity; this operation involves determining where the nomogram must be located on the wavenumber abscissa to yield the absorbance value under consideration. The nomograms used were the Dartnall A₁ nomogram¹³ for the rods, the frog green rod nomogram¹⁰ for the single cones and the P463 cones, and the rhesus monkey red cone spectrum¹⁷ for the P576 cones. For the short wave-sensitive cones, the maximum absorbance was estimated using 20 points on the right-hand side only of the provisional peak.



agreement with that given by Schwanzara¹² for an extract of the visual pigment of *Anableps*. The single cones contained a pigment maximally sensitive to short wavelengths ($\lambda_{\max} = 409$ nm), while the double cones contained two pigments absorbing at longer wavelengths, with maximum absorbances of 463 nm and 576 nm. One class of double cone contained only the long-wave-sensitive pigment in both members, whereas the other class contained both the middle- and long-wave-sensitive pigments (Fig. 1). The shape of the absorbance spectra of the rods was well fitted by a Dartnall nomogram¹³, which suggests that the pigment was probably based on vitamin A₁.

Ellipsosomes similar to those described by MacNichol *et al.*¹⁴ but termed 'oil droplets' by Borwein and Hollenberg⁵ were found in the ellipsoid region of the inner segment of the cones. The ellipsosomes in the double cones did not contain carotenoids, as found in birds and reptiles¹⁵, but contained a pigment whose spectrum closely resembled that of reduced

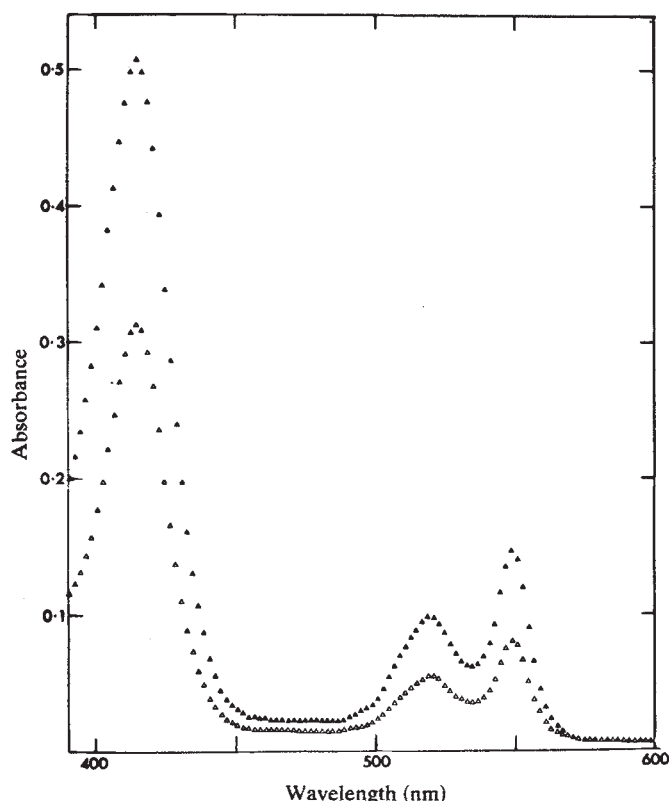


Fig. 2 ▲, Mean absorbance curve of six ellipsosomes from the P463 members of double cones and △, mean absorbance curve of seven ellipsosomes from the P576 members of double cones. The wavelengths of peak absorbance are 415, 519 and 549 nm.

cytochrome *c*, with an intense short-wavelength absorbance band ($\lambda_{\max} = 415$ nm) and two less intense absorbance bands at longer wavelengths (519 and 549 nm) (Fig. 2). The ellipsosomes of the double cones in which both members contained the 576-nm pigment (P576) were identical, with the short-wave absorbance band having a maximum density of ~0.3. A similar absorbance spectrum was measured for the ellipsosomes of the long-wave-sensitive member of the other class of double cone, but in the spectrum of the ellipsosomes of the members containing the P463, the density of the pigment was considerably greater, with a short-wave absorbance of ~0.5. Ellipsosomes were visible in the inner segments of the single cones sensitive to short wavelengths, but contained no detectable pigment.

As light must pass through the ellipsosomes to reach the photosensitive outer segment, it has been suggested that the ellipsosome acts as an intracellular colour filter¹⁴. The principal effect would be to filter a narrow band of violet-blue light (400–430 nm) from the outer segment. Making reasonable assumptions for the end-on absorbance of the cone outer

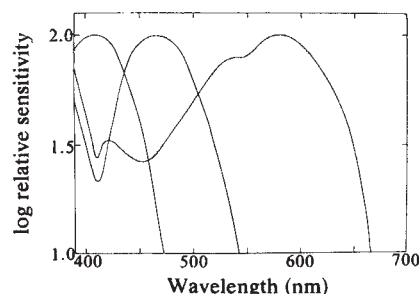


Fig. 3 Log relative sensitivity functions for the three classes of cone. These were derived by determining the absorbance spectrum for each class of cone and subtracting from this the mean absorbance spectrum of the ellipsosomes found in the inner segments of the corresponding cone type. In the case of the single cones, the sensitivity function is the same as the absorbance spectrum, as the ellipsosomes in these receptors are spectroscopically clear. The absorbance spectrum of a given class of cone (that is, the ratio of absorbed to incident light for a beam passing axially through the receptor) was calculated assuming an end-on absorbance of 0.25. The cone outer segments were ~6–10 μ m long but appeared to be broken at the tips, and we have taken the true outer segment length to be ~15 μ m. The specific absorbance of the cells was ~0.15, similar to values reported for other cones containing pigments based on vitamin A₁ (ref. 10).

segments, it is possible to construct relative spectral sensitivity functions for the different cone types (Fig. 3). It is clear that the filtering by the ellipsosomes narrows the short-wave limb of the visual pigment spectrum, particularly that of the P463 member of the double cone. This results in the P463 member of the double cone having a rapidly decreasing sensitivity from 460 nm to 420 nm, whereas the P409 single cones have a rapidly increasing sensitivity in the same region of the spectrum. This invites speculation as to possible neural interaction between these cone types which would confer increased wavelength discrimination in the short-wave region of the spectrum.

The Cyprinodontidae (flagfish, minnows) and the Poeciliidae (mollies, guppies) are closely related to the Anablepidae, all being members of the Order Microcyprini. The visual pigments of these families are very similar to those of *Anableps*; they too have violet-sensitive single cones, middle- and long-wave-sensitive double cones, and ellipsosomes in the double cone inner segments¹⁶. They also occupy a similar habitat in that they are all found very close to the surface in freshwater, and they are strongly diurnal and primarily surface feeders.

Although there seems to be no difference in the visual pigments in the two halves of the retina in *Anableps*, it cannot be excluded that the dorsal region may be more red-sensitive than the ventral region, either as a result of an uneven distribution of the various cone types in the two halves of the retina, or due to neural interaction giving prominence to the long wave-sensitive double cones.

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Serotonin alters the phosphorylation of specific proteins inside a single living nerve cell

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The large size and ready identifiability of molluscan neurones have made it possible to investigate the hypothesis^{1,2} that cyclic AMP-dependent protein phosphorylation may regulate neuronal electrical properties. For example, it has been found recently that intracellular application of the catalytic subunit of cyclic AMP-dependent protein kinase can alter the electrical activity of various molluscan neurones (refs 3, 4; J. De Peyer, H. Reuter and I.B.L., unpublished). We have recently demonstrated⁵ that injection of a specific protein kinase inhibitor into identified *Aplysia* neurone R15 blocks the increase in K⁺ conductance normally elicited in R15 by serotonin⁶, implicating protein phosphorylation in the regulation of a specific ion channel by a neurotransmitter. We report here attempts to identify phosphoproteins which may be involved in such regulation, by measuring protein phosphorylation in R15 following the intracellular injections of γ -³²P]ATP. The results indicate that we can indeed measure protein phosphorylation inside an individual living nerve cell, and that the extent of phosphorylation of several proteins in R15 is influenced by serotonin.

Previous attempts to measure protein phosphorylation in individual molluscan neurones have been carried out by incubating ganglia with ³²P_i, then isolating nerve cell bodies and analysing the radioactive phosphoproteins⁷⁻¹¹. These studies suffer from the major disadvantage that the cell body of interest can never be isolated entirely free of glia and portions of neighbouring neuronal cell bodies which contribute to the labelling pattern. Furthermore, only the cell body, and not the axonal and dendritic processes in the neuropile, is sampled by this procedure. We have circumvented these problems by developing techniques for the injection of 10⁵–10⁶ c.p.m. of γ -³²P]ATP into neurone R15, and have analysed the radioactive phosphoproteins produced. To examine the distribution of the radioactivity in the abdominal ganglion, we have fixed and sectioned ganglia following such injections, and have examined the sections by autoradiography. The results (not shown) demonstrate that all the radioactivity is restricted to R15. Thus, when the entire abdominal ganglion is analysed by gel electrophoresis, all the radioactive phosphoproteins originate from R15's soma and neuropile processes. R15 is voltage-clamped throughout the labelling period, so changes in the phosphorylation profile in the cell can be related to changes in membrane conductance. In addition, voltage-clamping assures that changes in phosphorylation are due directly to the effects of serotonin rather than to changes in the cell's spontaneous activity induced by serotonin.

Of the total radioactivity injected into neurone R15, ~2% was incorporated into trichloroacetic acid-insoluble, chloroform/methanol-washed material during the following 50 min (1.9 ± 0.6% in control (*n* = 14) cells, 2.1 ± 0.8% in serotonin-treated (*n* = 12) cells; not significantly different). Analysis of this material, after partial acid hydrolysis, using two-dimensional high-voltage thin-layer electrophoresis¹² shows that only two amino acid residues are phosphorylated, the major one being phosphoserine and the minor one phosphothreonine. This further substantiated that the extracted material was phosphoprotein. The polyacrylamide gel autoradiogram in Fig. 1 and the densitometric scans in Fig. 2

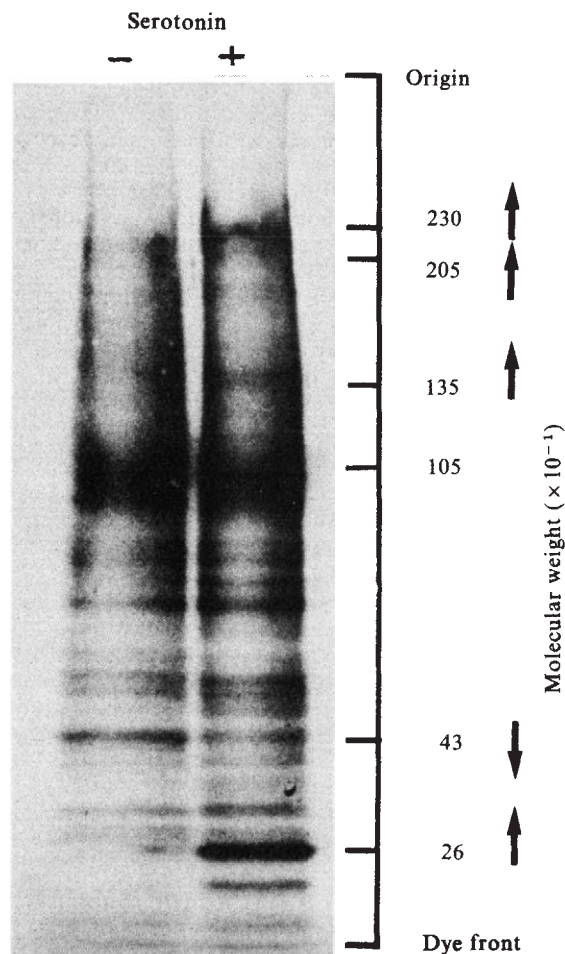


Fig. 1 Analysis of proteins phosphorylated in living neurone R15. Immediately before each experiment, $[\gamma$ -³²P]ATP (Amersham; >5,000 Ci mmol⁻¹) was concentrated in an Eppendorf microfuge tube, using a rotary evaporator, to a final concentration of 1 μ Ci ml⁻¹. For storage the $[\gamma$ -³²P]ATP was rediluted to a concentration of 50 mCi ml⁻¹ in H₂O and was kept at -20 °C. With this protocol the ATP preparation could be used several times without substantial radiolytic decay. Using a Hamilton syringe, ~50 nl of the concentrated ATP solution were placed in the tip of a microelectrode pulled from 'Microstar' capillary tubing (Radnoti Glass). Electrodes were used within minutes of filling, again to avoid substantial radiolytic decay and/or blockage of the electrode tip. ATP solution (0.5–2.0 nl; estimated by movement of the meniscus in the electrode tip) was injected into voltage-clamped neurone R15 (estimated cell body volumes 20–60 nl) under pressure. Analysis of the material remaining in the electrode tip after the injection indicated that >85% of the radioactivity was still in ATP. The total amount of ATP injected (0.1–0.4 pmol) was $\leq$ 1% of the endogenous level in R15 (D. Weinreich, personal communication). These ATP injections did not alter the current-voltage relationship of R15. The ganglion was first perfused with normal *Aplysia* saline for 30 min and then with either normal *Aplysia* saline or saline containing 5 μ M serotonin for an additional 20 min. Serotonin produces a large increase in K⁺ conductance in this time^{5,6}. Cells were voltage-clamped throughout, and only those exhibiting normal current-voltage characteristics and normal responses to serotonin were taken for analysis. Fifty minutes after injection the entire abdominal ganglion was frozen in liquid N₂, and homogenized in 1 ml of ice-cold stop buffer (50 mM MOPS, pH 7.0, 20 mM EDTA, 10 mM NaF, 1 mM ATP). The connective tissue sheath remained essentially intact and was discarded. Two ml of 20% trichloroacetic acid (TCA) was added to the homogenates at 4 °C. The resulting precipitates were collected by centrifugation, washed twice with 10% TCA, then twice with chloroform/methanol (1:2 then 2:1, v:v) to extract phospholipids. The pellets were redissolved in 100 μ l of sample buffer (100 mM dithiothreitol, 80 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Samples were boiled for 2 min and run on an SDS-polyacrylamide gradient (5–17.5% acrylamide) slab gel¹³. In this experiment, the control (-serotonin) track was loaded with 11,600 c.p.m., incorporated following an injection of 360,000 c.p.m. of $[\gamma$ -³²P]ATP into R15; the +serotonin track contained 18,000 c.p.m. (600,000 c.p.m. injected). Following electrophoresis the gel was dried and autoradiographed using Kodak X-Omat X-ray film for 7 days. The resulting autoradiogram exhibits at least 15 phosphoprotein bands originating from within R15. $\uparrow$, Bands whose phosphorylation state was significantly increased or decreased, respectively, by serotonin (see Table 1).

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Table 1 Relative incorporation into individual phosphoprotein bands

M_r	% Of total radioactivity		Mean % change
	Controls (n = 8)	Serotonin (n = 8)	
230,000	4.1 ± 0.3	6.9 ± 1.0	+69*
205,000	7.5 ± 0.5	10.3 ± 1.1	+41*
135,000	8.2 ± 0.5	10.6 ± 0.7	+29*
105,000	19.7 ± 1.5	20.2 ± 1.6	+2
82,000	8.7 ± 0.6	8.6 ± 0.7	-1
68,000	11.8 ± 0.8	13.1 ± 1.1	+12
50,000	8.8 ± 0.5	8.8 ± 0.3	0
43,000	5.0 ± 0.2	4.1 ± 0.2	-21*
36,000	4.4 ± 0.5	4.2 ± 0.6	-5
31,000	3.9 ± 0.8	2.7 ± 0.3	-30
26,000	3.5 ± 0.3	5.9 ± 0.5	+55*
22,000	5.8 ± 1.2	3.6 ± 0.4	-31

Densitometric scans of autoradiograms (for example, Fig. 2) were used to quantify the relative amount of radioactivity in individual phosphoprotein bands. Areas under each peak were computed as a percentage of the total area of the scan; thus, the numbers in the table represent the radioactivity in each band as a percentage (means ± s.e.m.) of the total radioactivity in the gel. Experiments performed on the same day were paired and Student's *t*-test used to evaluate the differences (expressed here as mean % change) between control and serotonin-treated ganglia. Although the 31,000- and 22,000- M_r proteins showed large mean per cent changes, they also exhibited large variability (see, for example, Fig. 2) and thus these changes were found not to be significant ($P > 0.6$).

*Changes induced by serotonin which were significant ($P < 0.02 < 0.007$).

demonstrate that at least 15 phosphoproteins, of molecular weights (M_r s) 22,000–230,000, were labelled in R15 during the 50 min following injection of [γ - 32 P]ATP. When serotonin was added to the medium bathing the abdominal ganglion during the last 20 min of the labelling period, there was a large increase in K^+ conductance in R15^{5,6}, and a change in the extent of labelling of several phosphoproteins. This number of changes is not surprising because serotonin increases cyclic AMP levels in this cell⁶ and this could lead to a variety of changes in the phosphorylation state of many intracellular proteins. The phosphorylation of several high molecular weight proteins (M_r s 230,000; 205,000; 135,000) and of a 26,000- M_r protein was increased significantly in the presence of serotonin (Figs 1, 2;

Table 1). In addition, there was a decrease in the phosphorylation of a 43,000- M_r band (Figs 1, 2; Table 1).

There is only limited information on protein phosphorylation in the *Aplysia* nervous system. Although our early studies^{7,8} (recently confirmed by Paris *et al.*¹⁰) indicated that serotonin could influence the phosphorylation state of a high molecular weight protein, it is not clear whether it is related to the 135,000- M_r protein described here. The present experiments indicate that, in addition to this phosphoprotein, the phosphorylation of several other proteins in R15 can be influenced by serotonin. This *in vivo* approach should permit an analysis of the possible role of these phosphoproteins in the regulation of K^+ conductance in this cell.

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Neuropeptide coexistence in human cortical neurones

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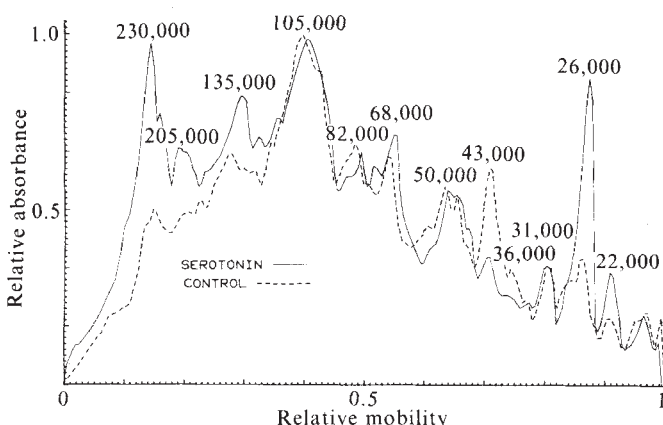


Fig. 2 Densitometric scans of the autoradiograms shown in Fig. 1. Autoradiograms were scanned using a Joyce-Loebl densitometer. The scanner was centred on each gel track, and the scan width was set at maximum to average the density across the entire track. Because the 105,000- M_r peak was not affected by serotonin (see Table 1), we normalized the scans to the height of this peak to facilitate visualization of the specific effects of serotonin. This analysis permits the evaluation of changes in labelling of specific phosphoproteins independent of differences in total incorporation from one cell to another. —, Serotonin; ----, control.

The growth hormone release-inhibiting factor somatostatin was originally isolated and characterized from hypothalamic extracts¹. Avian pancreatic polypeptide (APP) was first isolated from chicken pancreas². Using antibodies raised against these peptides, both somatostatin-like^{3–5} and APP-like^{6,7} immunoreactivities have been found in many regions of the nervous system, and the peptides have been shown to coexist with catecholamines or other neuropeptides in certain neuronal systems^{8–11}. In particular, we have recently reported that some neurones in the rat telencephalon contain both somatostatin- and APP-like immunoreactivities¹². We present here immunohistochemical evidence that in the human cerebral cortex also, some neurones contain both immunoreactivities.

Specimens of human frontal, temporal and cingulate cortex were obtained from patients undergoing lobe resection as a treatment for glial tumours. Small pieces (about 10 × 5 × 2 mm) of cortex of normal appearance were taken from cortical areas, located as far as possible from the pathological area, immediately after the lobe was resected. The specimens were immersed in ice-cold 10% formalin in 0.1 M phosphate buffer for 2–6 h. The time between occlusion of the blood supply to the cortex and the immersion of the tissue was ~30 min. After fixation

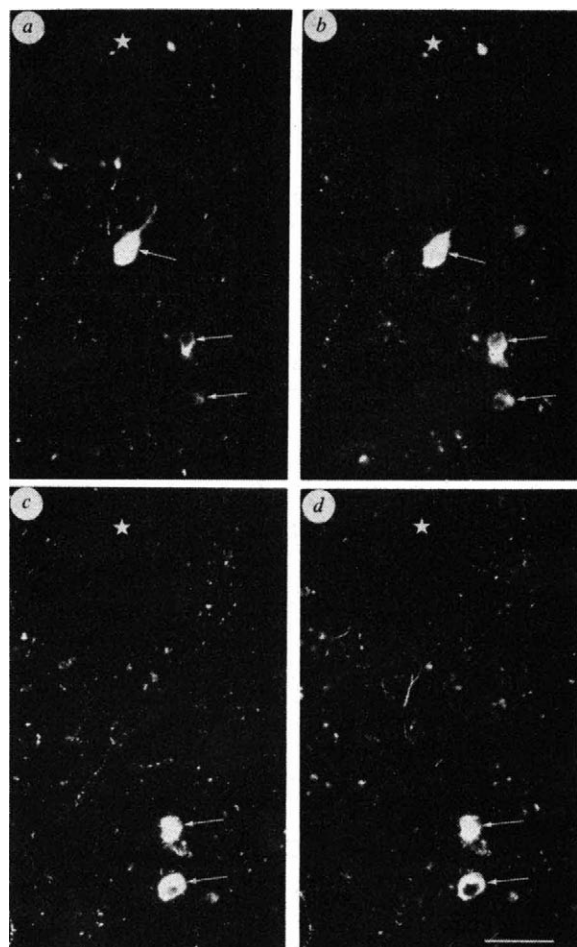


Fig. 1. *a-d*, Immunofluorescence micrographs of 10 μ m thick sections of the human neocortex after incubation with antisera to somatostatin (*a, d*) or APP (*b, c*). *a* and *b*, The same section stained initially for somatostatin (*a*), then eluted according to Tramu *et al.*¹⁶ and restained with antibodies against APP (*b*). *c*, *d*, The same section, which is adjacent to that shown in *a* and *b*, first stained for APP-like immunoreactivity (*c*), then eluted¹⁶ and restained for somatostatin (*d*). Arrows indicate neurons that contain both somatostatin- and APP-like immunoreactivities, in either the serial section or the restaining experiments. ☆, A blood vessel. Scale bar, 50 μ m for *a-d*.

the tissues were rinsed in 0.1 M phosphate buffer containing 5% sucrose for at least 24 h. Sections were then cut on a cryostat (Dittes) and processed according to the indirect immunofluorescence technique of Coons and collaborators^{13,14}.

Alternate 10 μ m thick sections of cortex were incubated overnight at 4 °C with antisera raised in rabbits against APP^{7,12} or somatostatin¹⁵. Both sera were diluted 1:100 in 0.1 M phosphate buffer containing 0.3% Triton X-100. After rinsing, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dako), rinsed, coverslipped and examined in a Zeiss fluorescence microscope.

In further experiments, sections that had been stained for one neuropeptide were photographed, then the initial antiserum was eluted with 0.1% KMnO_4 in 0.2% $\text{H}_2\text{S}_2\text{O}_4$ for 30 s and the sections decolorized in 0.5% $\text{Na}_2\text{S}_2\text{O}_5$ for 15 s (ref. 16). The sections were then incubated in the FITC-conjugated IgG and examined in the fluorescence microscope. If no staining was observed at this stage, indicating that the first antibody had been successfully eluted, the sections were then incubated with antibody to the other neuropeptide, followed by the FITC-conjugated IgG and once more examined in the microscope and photographed. Using this procedure the same section could be sequentially stained for somatostatin- and APP-like immunoreactivities.

Antisera incubated with an excess of the respective neuropeptide (50 μ g of synthetic somatostatin or APP per ml antiserum diluted 1:10) served as controls. Preabsorption of the somatostatin antibody with APP or of the APP antibody with somatostatin had no effect on the resultant staining pattern. This indicates that none of the sera used in these experiments recognizes both peptides.

In sections incubated with antisera to APP, we observed a dense network of immunoreactive fibres. In specimens cut perpendicular to the cortical surface, a definite pattern of fibres could be discerned. In cortical layers II–VI, most of the APP-positive fibres appeared to run vertically out from the deeper layers towards the surface, although many fibres branched laterally as well. In contrast, in layer I, most of the APP-positive fibres ran parallel to the cortical surface; however no fibres were seen in the outer 50 μ m of cortex. This staining pattern was observed in frontal, temporal and cingulate cortex specimens.

In sections incubated with anti-somatostatin antisera, the pattern of fibres observed was similar to that described for APP. The somatostatin-positive fibres fluoresced much more weakly, however, and, in particular, the fibres running parallel to the cortex in layer I were much less heavily stained.

Many immunoreactive cell bodies were observed in the cortex following incubation of the sections with either APP or somatostatin antisera. The cells were scattered throughout layers II–VI, and were also commonly found in the subcortical white matter. The cells were pyramidal, bipolar, round or multipolar in shape, with dendrites spreading out in all directions.

In serial sections stained alternatively for APP or somatostatin, we often found that one portion of a cell was positive for APP in one section while in a second section, the other portion was positive for somatostatin (Fig. 1*a, c*). This indicates that a single cell can contain both neuropeptides. Examples of this were found in all cortical layers except layer I.

The coexistence of somatostatin- and APP-like immunoreactivities in a cortical neurone was also frequently demonstrated in the elution–restaining experiments. Using this protocol, we found neurones that first stained for somatostatin (Fig. 1*a*) and then, after elution, restained for APP (Fig. 1*b*), and cells that were first shown to be APP positive (Fig. 1*c*) and, after elution and restaining, also seen to contain somatostatin immunoreactivity (Fig. 1*d*).

The occurrence of more than one neuroactive substance in a single neurone has recently been reviewed^{17,18}. The phenomenon was first observed in invertebrates, but has since also been observed in mammalian neurones, and now in human brain.

We have shown that the coexistence of somatostatin and APP reported here is not due to cross-reactivity of the antisera. Also, larger presumed precursor molecules have been found for both peptides^{19–22} and they seem quite distinct. This and the clear differences in the distribution of the peptides in other brain regions¹² suggests that they do not have a common precursor and that their coexistence is a special property found only in certain cell types.

The coexistence of somatostatin- and APP-like peptides within a selective population of neurones suggests that they both may function as transmitters or modulators of neural information. In this regard, somatostatin has been found to have potent electrophysiological effects on cortical neurones^{23–25} and both calcium-dependent release^{26,27} and high-affinity binding²⁸ of somatostatin have been demonstrated in mammalian brain. APP has also been found to have postsynaptic actions²⁹. Thus the presence of these two neuroactive substances in a single cortical neurone may allow the cell to transmit more than one message to its postsynaptic targets.

Recently somatostatin levels have been found to be reduced in the cerebrospinal fluid of patients with Huntington's disease³⁰. Also, cortical somatostatin levels are markedly reduced in Alzheimer's disease and in senile dementia of Alzheimer type^{31,32}. This suggests that a loss of forebrain somatostatin

neurones may be a symptom of these diseases. As an APP-like peptide is contained in many forebrain somatostatin cells, these diseases are probably also associated with a loss of APP-like immunoreactivity. The role of cortical neurones containing both somatostatin- and APP-like immunoreactivity in these conditions remains to be determined.

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Fission-spectrum neutrons at reduced dose rates enhance neoplastic transformation

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We have studied the effect of reduced dose rates of radiation on neoplastic transformation using the C3H/10T1/2 mouse embryo-derived cell line developed by Reznikoff *et al.*¹. In this cell system, transformation can be measured by scoring the foci of piled-up cells that occur after the loss of contact inhibition of growth. We report here that reduction of the dose rates of fission-spectrum neutrons (of mean energy 0.85 MeV) from 38.5 or 10.3 to 0.43 and 0.086 rad min⁻¹ has no effect on cell killing but increases transformation frequency. These data are in contrast to previously published results obtained with ⁶⁰Co γ rays where reduction of the dose rate resulted in a marked reduction in transformation frequency^{2,3}. Although cancer induction in man involves many factors besides those at the cellular level, the implication from our findings is that the risk of cancer induction due to work-related exposure to neutrons in the nuclear power industry may be greater than previously thought.

Cell cultures were grown at 37°C in plastic T-flasks, or in plastic 100-mm Petri dishes, in Eagle's basal medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Reheis Chemical Co., Illinois) according to methods previously described^{2–5}. The JANUS reactor of the Division of Biological and Medical Research, Argonne National Laboratory, was used to expose cells to fission-spectrum neutrons⁵. A special incubator, made of light plastic and styrofoam to minimize any changes in the quality of the neutron beam, was used for the low dose rate exposures at 0.43 and 0.086 rad min⁻¹, and the cells were kept at 37°C while attached in plastic T-flasks containing Eagle's supplemented medium. In all experiments, irradiated cells were removed from the incubator after exposure, trypsinized, plated out in Petri dishes and placed in

conventional incubators for the 6 weeks necessary for transformed foci to develop^{1,5}. In a control experiment, cells were grown in the special incubator without irradiation. No transformed colonies were observed for these controls; that is, the transformation frequency per surviving cell was $<8.7 \times 10^{-5}$. Dosimetry was performed inside the incubator at the position of the cells⁵. High-dose rate irradiations (38.5 or 10.3 rad min⁻¹) were carried out at room temperature⁵. Transformed foci were scored as previously described^{1,5} and transformation frequencies per surviving cell were calculated according to Han and Elkind⁵.

Consistent with the earlier results of Sinclair⁶ with V79 Chinese hamster cells, the data in Fig. 1 show no significant variation in cell killing after a reduction in the fission-spectrum neutron dose rate from 38.5 to 0.086 rad min⁻¹ (5.2 rad hr⁻¹) even though the cells were irradiated in optimal growth conditions. The lowest dose rate used here was 20-fold less than that previously reported^{6,7}. At 0.086 rad min⁻¹, cells undergo one

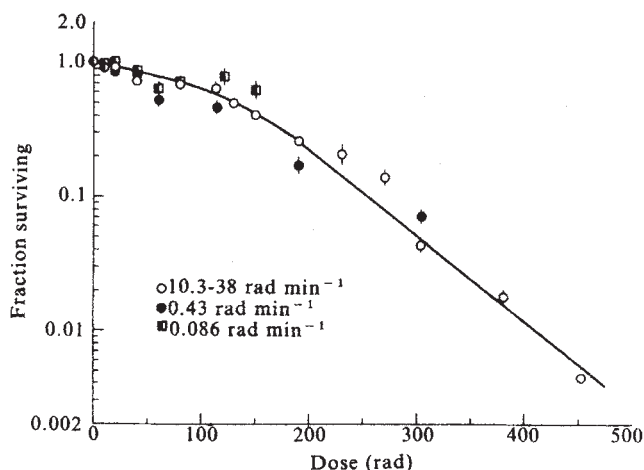


Fig. 1 The survival of 10T1/2 cells exposed to fission-spectrum neutrons from the JANUS reactor at the Argonne National Laboratory. The line is drawn through the average surviving fractions from all experiments performed over a 3-yr period. The individual data points shown at high and low dose rates were obtained concurrently as part of this study; the doses refer to the integrated exposures of populations of cells. Cells in growth medium were exposed at 37°C and immediately after exposure were suspended by trypsinization and plated for survival. Plating efficiency = 21–42%, multiplicity ~ 1. Bars represent standard errors⁵.

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Table 1 Transformation of 1OT1/2 cells by fission-spectrum neutrons

Dose (rad min ⁻¹)	Surviving fraction	No. surviving cells per dish*	No. of dishes	No. of foci†	No. dishes without foci	λ	Transformation frequency per viable cell ($\times 10^{-4}$)
Controls	1.0	242	2,409	6	2,403	0.0025	0.103
10.3–38.0							
10	0.970	135	173	2	171	0.0116	0.86
21	0.957	212	165	5	160	0.0308	1.4
41	0.753	200	119	8	113	0.0517	2.6
76	0.709	176	112	17	97	0.143	8.2
114	0.651	404	19	7	12	0.379	9.4
151	0.412	108	15	5	10	0.405	37.5
189	0.269	223	68	71	22	1.13	50.6
227	0.172	536	16	16	3	1.67	31.2
303	0.0421	107	19	9	12	0.460	42.9
378	0.0178	299	56	91	12	1.54	51.5
454	0.00450	250	20	23	4	1.61	64.4
0.43							
10	0.920	150	166	34	146	0.128	8.6
21	0.864	167	356	45	322	0.10	6.0
41	0.831	170	262	31	232	0.122	7.1
62	0.540	281	19	8	12	0.459	16.3
114	0.462	203	144	43	108	0.287	14.1
189	0.171	90	103	40	74	0.331	36.3
303	0.070	129	83	54	41	0.588	110.0
0.086							
10	0.941	145	188	19	172	0.089	6.1
21	0.961	136	228	24	205	0.106	7.8
41	0.869	169	164	28	139	0.165	9.7
62	0.655	187	141	26	121	0.152	8.2
80	0.716	173	12	4	8	0.405	23.4
121	0.823	195	17	11	10	0.531	27.2
150	0.647	170	9	10	4	0.811	47.6

A survival curve based on averages of the data from all experiments (Fig. 1) was used to estimate surviving fractions for the exposures at 10.3–38.0 rad min⁻¹. For the low-dose rate exposures, surviving fractions are those determined from the same population used to estimate the transformation frequency. Details of the λ calculation and its standard error are given in ref. 5. λ , The average number of transformed foci per dish, was computed from the proportion of dishes free of transformed colonies, f , by $\lambda = -\ln f$. Transformation frequency = λ /number of surviving cells per dish. Controls, that is, unirradiated cells, represent the cumulative data from the transformation experiments reported for this and other studies^{2–5}.

* Number corrected for plating efficiency of unirradiated cells and surviving fraction per 90-mm plastic Petri dish.

† Total number of type 2 and 3 transformed foci^{1,5}.

or two divisions during exposure, depending on the length of exposure. However, flow cytofluorographic analyses of the DNA content of the cells in each sample, using an Ortho Instruments 2150 system, indicated that no significant perturbation in distribution of cell cycles occurred until 150 rad had accumulated at 0.43 rad min⁻¹, or 100 rads at 0.086 rad min⁻¹. Thus, it is unlikely, especially at the smaller doses, that our results were significantly affected by changes in cell-cycle distributions during exposure.

There was no apparent reduction in transformation frequency when the dose rate of fission-spectrum neutrons was reduced by a factor of 200 (38.5 to 0.086 rad min⁻¹). Indeed, whereas at higher doses the high and protracted dose rates seemed to induce the same transformation frequency, at 10–70 rad there was a significant enhancement of transformation frequency for dose rates of 0.43 and 0.086 rad min⁻¹. The data for transformation at high dose rates in Fig. 2 are a combination of those previously published⁵ and those obtained in the present series of experiments; the two sets of results did not differ significantly and therefore were pooled. Table 1 summarizes the results plotted in Figs 1 and 2.

Although cells apparently are unable to repair sublethal damage when exposed to the JANUS reactor beam at reduced dose rates, consistent with earlier studies performed with the same beam using fractionation of high dose-rate exposures^{8,9}, the enhanced transformation shown in Fig. 2 is unexpected. Han and Elkind⁵ showed that the fractionation of high dose-rate exposures of neutrons from the JANUS reactor had little if any effect on transformation frequency per surviving cell. Figure 2 shows that reduced dose rates significantly enhance transformation in the low dose region from 10–70 rad, with no concomitant effect on survival (Fig. 1).

To illustrate further the qualitatively unexpected nature of

our transformation results, Fig. 3 compares the ratios of transformation frequencies per survivor at low to high dose rates for neutrons from the JANUS reactor and for ⁶⁰Co γ rays³. Clearly, the reduced dose rates of γ -ray irradiation, in the 0–100 rad region, are less effective than high dose rates, while for the same dose interval, low dose rates of fission-spectrum neutrons are appreciably more effective than high dose rates. Furthermore, few if any of the changes in transformation frequency can be attributed neither to differential cell killing, in view of the small amount, and dose rate independence of lethality in this dose range (Fig. 1), nor to parasynchronous effects as already noted.

There are some indications, primarily from results obtained with *in vivo* systems^{10–13}, that low dose rates of fission-spectrum neutrons may be more effective at producing tumours than high dose rates. However, interpretation of the results following whole-body exposures is complicated by the interactions among different tissue and organ systems (for example, see Fry¹² in reference to the induction of Harderian gland tumours). Our results, obtained with actively growing mammalian cells in culture, show that enhanced induction rates of neoplastic transformation could be due to intracellular events in the target cells themselves. A similar specificity applies to the observation that reduced transformation frequencies due to low dose rates of ⁶⁰Co γ rays result from repair processes in the target cells³.

Finally, we may draw from our data several inferences about mechanisms. Compared with γ rays, fission-spectrum neutrons may be considered a high linear energy transfer (LET) radiation. The shoulder on the neutron survival curve indicates that accumulation of sublethal damage contributes to cell killing and that such damage is not repaired, in contrast to the repair of sublethal damage from γ rays observed for prolonged exposure³. As an acute dose of fission-spectrum neutrons does

not prevent the repair of the damage that is additive to X-ray-induced sublethal damage⁸, we infer that different targets are affected by high as opposed to low LET radiation and that the damage (or the target) responsible for neutron-induced killing cannot be repaired. With respect to transformation, we note that, at reduced dose rates of γ rays, survival increases but transformation per surviving cell decreases, both relative to high dose rate exposure, and that transformation is reduced even in the dose region where cell killing is minimal. In similar exposure conditions, reduced dose rates of fission-spectrum

neutrons result in enhanced transformation. Thus we infer that the repair of γ -ray-induced transformation damage has a net error-free component of repair, and that the increase in neutron-induced transformation results from a net error-prone component of repair independent from sublethal damage in view of the dose rate independence of the latter. Further experiments are in progress to determine whether the same targets are involved in transformation induced by high- compared with low-LET radiations.

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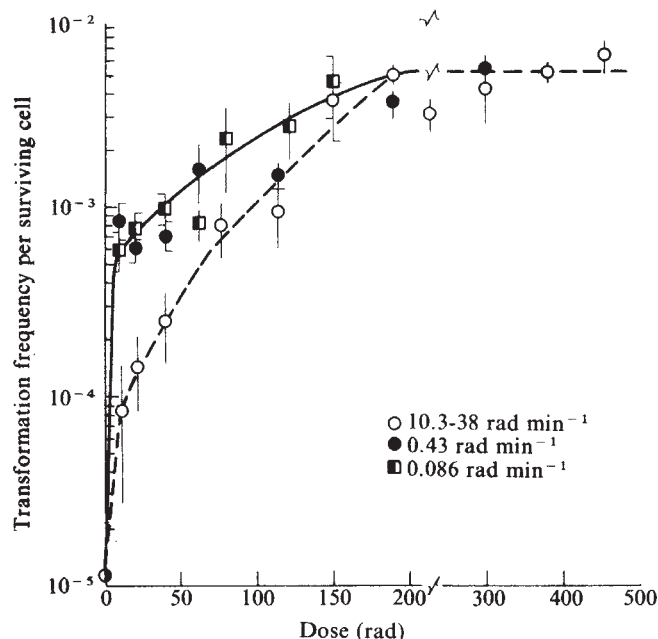


Fig. 2 Transformation frequency per surviving cell for 10T1/2 cells exposed to fission-spectrum neutrons (data given in Table 1). The broken line represents the acute dose rate curve and the solid line indicates the data from both low dose rates used. Other details as for Fig. 1.

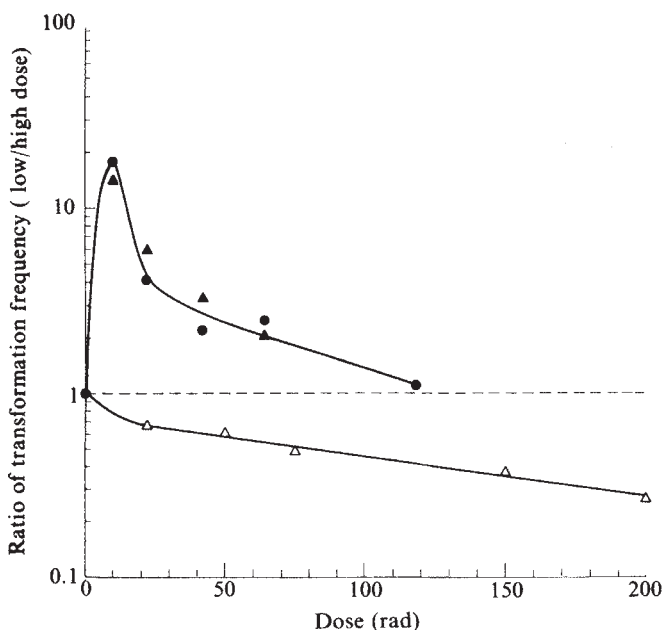


Fig. 3 The ratio of transformation frequency per surviving cell for low compared with high dose rates, obtained for 10T1/2 cells, as a function of dose. For both fission-spectrum neutrons and ^{60}Co γ rays, the doses refer to the integrated exposures of populations of cells. Dose rate independence would plot as the broken line shown. $\bullet$, $\blacktriangle$ = 0.43 and 0.086 rad min^{-1} , respectively, of JANUS neutrons. Δ , 0.1 rad min^{-1} ^{60}Co γ rays.

Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA

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Human adenoviruses can morphologically transform rodent cells *in vitro*. The transforming gene(s) resides in the early region 1 (E1) transcription unit (0-11.2 map units) at the left end of the conventional adenovirus genome¹⁻⁴. The only adenovirus-transformed human cell line reported so far, the 293 cell line, was isolated from cell cultures transfected with sheared DNA of the non-oncogenic subgroup C adenovirus type 5 (Ad 5)^{5,6}. The ability of this line to complement the growth of Ad 5 E1 mutants has facilitated the isolation of transformation-defective Ad 5 mutants^{7,8}. As no oncogenic subgroup A adenovirus type 12 (Ad 12) transformed human cell lines were available for the selection of Ad 12 E1 mutants, we attempted to isolate such lines by transfecting cell cultures with a recombinant plasmid (pAsc2) which contained the Ad 12 EcoRI-C DNA fragment (0-16.5 map units) inserted at the EcoRI site of pAT153 (ref. 9). We describe here the isolation of a pAsc2-transformed human embryo retinoblast cell line that contains many copies of the Ad 12 transforming region, expresses virus-specified proteins and, following inoculation into athymic mice, forms tumours which resemble retinoblastomas.

The suggestion that Ad 12-induced rodent tumours originate specifically from primitive neural epithelium¹⁰, together with the discovery that this virus induces retinal tumours following intraocular inoculation of rats and baboons^{11,12}, prompted us

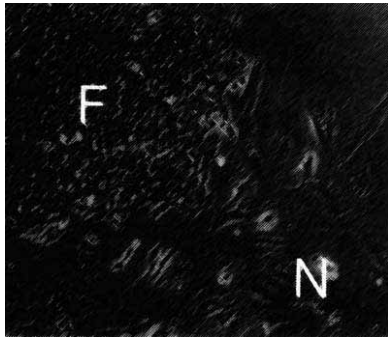


Fig. 1 An edge of a pAsc2-transformed human embryo retinoblast focus. The retinas from the eyes of an 18-week-old male fetus (therapeutically aborted) were distributed in six 50-mm tissue culture dishes and grown in RPMI medium containing 20% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin, 100 units ml^{-1}). Ten days after seeding, the cultures (containing $\sim 1 \times 10^6$ cells per dish) were transfected with pAsc2 (10 μg per dish) using the calcium phosphate precipitation technique²⁵ with the glycerol boost²⁶. Seventeen hours after transfection, each culture, including two control cultures which were transfected with pAT153 alone (10 μg per dish), was subcultured to four 50-mm dishes. The cells were then grown in RPMI medium containing 5% FCS and antibiotics. Only one pAsc2-transformed focus (F) was identified against the background of normal (N) cells; the experiment was terminated 10 weeks after transfection. This focus was isolated and established in a 35-mm dish and after 14 days the transformed cells were transferred to a 50-mm dish. This transformed cell line (Ad 12 HER1) was subsequently grown and passaged in 90-mm dishes at a split ratio of 1:15; the cells are maintained in RPMI containing 10% FCS and antibiotics. $\times 75$.

to attempt to transform human embryo retinoblasts (HER cells) *in vitro* with pAsc2. Primary cultures of human retinoblasts were transfected with pAsc2, and one transformed focus, identified 45 days after transfection (Fig. 1), was isolated and established as a continuous cell line. This cell line has been designated Ad 12 HER 1 and is the first reported Ad 12-

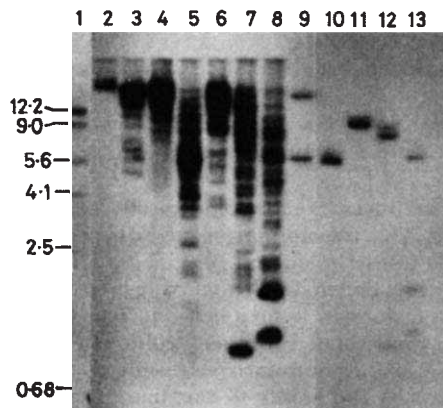


Fig. 2 Detection of Ad 12 DNA sequences in Ad 12 HER1 cell DNA. Samples (5 μg) of Ad 12 HER1 DNA were digested with restriction endonucleases, electrophoresed in a 0.8% agarose gel and subjected to Southern blotting analysis as described previously⁹. Track 1, 5 μg normal HEK cell DNA and 20 pg Ad 12 DNA (equivalent to ~ 0.64 copies per cell) digested with *EcoRI*; track 2, high molecular weight Ad 12 HER1 DNA; tracks 3, 4, 5, 6, 7, 8, Ad 12 HER1 DNA digested with *BglII*, *SstI*, *EcoRI*, *BamHI*, *XbaI* and *HindIII* respectively; tracks 9, 10, 11, 12, 13, 5 μg normal HEK cell DNA and 20 pg pAsc2 (equivalent to 2.4 copies per cell) digested with *BglII*, *EcoRI*, *BamHI*, *XbaI* and *HindIII* respectively. Track 1 was hybridized with ^{32}P -labelled Ad 12 DNA; tracks 2-13 were hybridized with ^{32}P -labelled Ad 12 *EcoRI*-C DNA fragment. The sizes of the Ad 12 DNA *EcoRI* fragments (in kilobase pairs) are indicated. Summation of the autoradiographic bands and their intensities indicates that each Ad 12 HER1 cell contains the equivalent of ~ 50 copies of the Ad 12 *EcoRI*-C DNA fragment.

transformed human cell line. Cytogenetic analysis revealed a pseudotetraploid human karyotype. We have grown the Ad 12 HER1 cell line beyond the 36th tissue culture passage level and at no time has its growth entered a crisis phase. This is in contrast to the establishment of the 293 cell line⁶.

Human embryo retinoblasts are the only human cell type we have been able to transform reproducibly with pAsc2. In two recent experiments, 10 transformed cell foci were identified in seven dishes of pAsc2-transfected HER cells (10 μg pAsc2 per 1×10^6 cells per dish); 4 of these foci were isolated and established as cell lines. The low specific transforming activity of pAsc2 on HER cells, which is an order of magnitude lower than on cultures of primary rat cells⁹, is >100 -fold higher than that on human embryo kidney (HEK) cells. The isolation and biological properties of the pAsc2-transformed HEK cell lines and additional pAsc2-transformed HER cell lines will be reported elsewhere (J. Whittaker, P.B. and P.H.G. in preparation).

When Ad 12 HER1 cell DNA was digested with the restriction endonucleases *BglII* or *SstI* and subjected to Southern blotting analysis¹³ using ^{32}P -labelled Ad 12 *EcoRI*-C DNA fragment as probe, Ad 12 sequences were found on many DNA fragments (Fig. 2, tracks 3 and 4, respectively) whose sizes

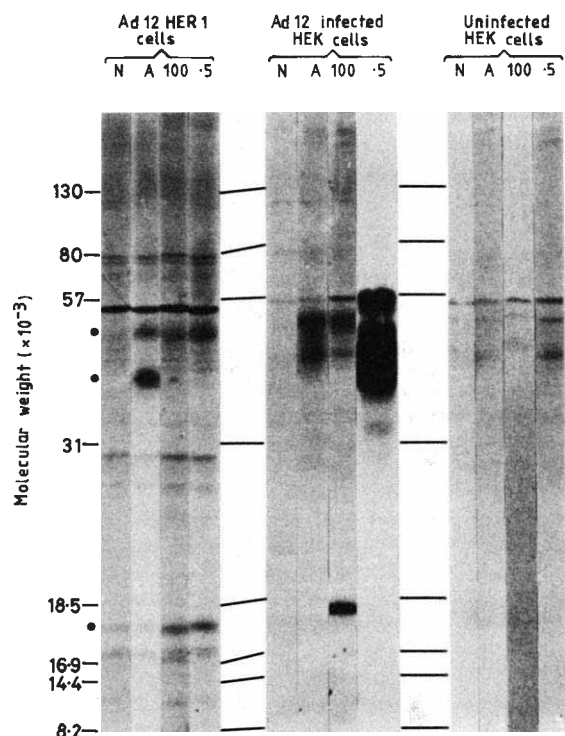


Fig. 3 Immunoprecipitation of Ad 12-specific proteins. Ad 12 HER1 cells, HEK cells or Ad 12-infected HEK cells (15 h after infection at M.O.I. of 400 plaque-forming units per cell) were preincubated in 9-cm dishes for 1 h in 3 ml of methionine-free modified Eagle's medium, containing 2% (v/v) FCS, and then labelled for 2 h in the same medium containing 100 μCi L- ^{35}S -methionine (>600 Ci mmol^{-1} , Amersham). Cleared extracts were made from the labelled cells and aliquots were immunoprecipitated with normal rat serum or Ad 12 tumour-bearer serum (pAsc2 HLBK1, Ad 12/LP/100-1 or 0.5J4) essentially as described elsewhere²⁷. Immunoprecipitates were run on 10-15% gradient SDS-polyacrylamide gels²⁸ and ^{35}S -labelled proteins were detected by fluorography²⁹. The cell extracts and tumour bearer sera are identified above the tracks; normal rat serum and rat sera raised against pAsc2 HLBK1, Ad 12/LP/100-1 or 0.5J4 are identified as N, A, 100 and .5 respectively. •, Ad 12-specific proteins expressed in Ad 12 HER1 cells. The positions and sizes of molecular weight standards are indicated to the left of the tracks containing extracts of Ad 12 HER1 cells; their positions in the tracks containing extracts of uninfected and Ad 12-infected HEK cells are indicated by lines connecting to the Ad 12 HER1 tracks.

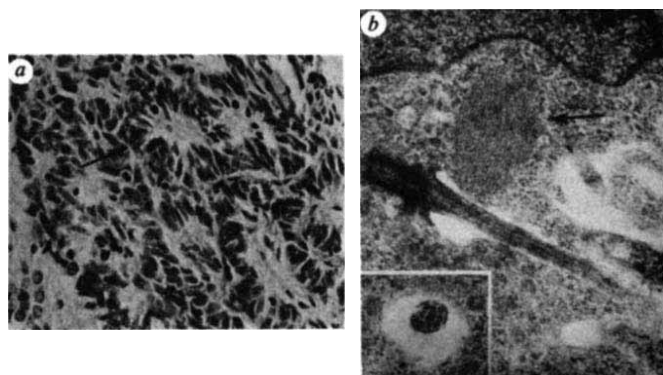


Fig. 4 *a*, Section through a cerebral tumour produced in a nude mouse following intracranial inoculation of Ad 12 HER1 cells. The arrow indicates a Homer-Wright rosette. The tumour was fixed in FAM (formaldehyde, acetic acid and methanol (1:1:8), paraffin sectioned and stained with haematoxylin-eosin. $\times 650$. *b*, Electron micrograph of part of an Ad 12 HER1 cell showing a longitudinal section through a cilium and a multilamellar structure (arrow) which is reminiscent of the outer segment of a photoreceptor cell ($\times 31,250$). Inset, transverse section of an Ad 12 HER1 cilium showing 9+0 microtubule configuration ($\times 75,000$). Cells were fixed in 1% glutaraldehyde and post-fixed with 1% osmium tetroxide. Sections were stained with uranyl acetate and lead citrate.

ranged from >12 kilobase pairs (12 kb) to 2.2 kb. As these enzymes cleave host DNA sequences only, each fragment represents a separate insert of pAsc2 sequences. DNA fragments which co-migrated with the Ad 12 *EcoRI*-C DNA fragment and linear pAT153 DNA (data not shown) following digestion of Ad 12 HER1 DNA with *EcoRI* (Fig. 2, track 5) produced intense autoradiographic bands. This suggests that Ad 12 HER1 cells contain intact, integrated copies of the viral and vector sequences and it is likely that some of the inserts contain tandem repeats of the entire recombinant plasmid. The Ad 12 and Ad 12-pAT153 DNA fragments expected from digestion of pAsc2 by *EcoRI*, *BamHI*, *HindIII* or *XbaI* were all identified in Ad 12 HER1 DNA following digestion with these enzymes (Fig. 2, tracks 5, 6, 7, 8). In addition, other fragments were identified which contained both Ad 12 DNA and pAT153 or these DNA species independently. The structures of these fragments, ranging in size over >12–0.63 kb, have not been determined.

^{35}S -methionine-labelled extracts from Ad 12 HER1 cells or HEK cells collected 18 h after infection by purified Ad 12 virus were immunoprecipitated using Ad 12 tumour-bearer rat sera. The sera were raised against tumorigenic cell lines that expressed proteins from Ad 12 early region 1 and other early regions (0.5J4)¹⁴ or from E1 only (Ad 12/LP/100-1 (ref. 15) and pAsc2 HLBRK1 (ref. 9)). Polypeptides with molecular weights of 52,000 (52K), 41K and 18K were detected (Fig. 3) and as they were not immunoprecipitated from extracts of uninfected HEK cells, must be authentic Ad 12-specified proteins. Therefore, Ad 12 HER1 cells express proteins which are known to be encoded within the Ad 12 E1 transcription unit^{16,17}.

The tumorigenic potential of Ad 12 HER1 cells has been ascertained by inoculating 6–10-day-old congenitally athymic nude mice. Subcutaneous inoculation of 5×10^6 Ad 12 HER1 cells at the fifth, sixth and seventh tissue culture passage levels resulted in tumours at the site of inoculation in 1/7, 1/5 and 1/6 injected animals respectively, with latent periods of 73–89 days. However, all animals (4/4) injected intracranially with as few as 1×10^4 cells at the eighth passage died from cerebral tumours with a mean time to death of 75 days. Thus, Ad 12 HER1 cells are highly tumorigenic when inoculated intracranially. Histological analysis of Ad 12 HER1-induced tumours showed them to consist of clusters of small cells with many mitotic figures. Homer-Wright rosettes and incomplete rosettes were frequently seen (Fig. 4a); these rosettes and

Flexner-Wintersteiner rosettes are characteristic of poorly differentiated human retinoblastomas¹⁸.

Electron microscopic analysis occasionally revealed cilia with 9+0 microtubule configuration on Ad 12 HER1 cells (Fig. 4b and inset). This configuration is characteristic of cilia of normal human retinal photoreceptor cells and retinoblastoma cells¹⁹ and has also been observed in cilia of Ad 12 virus-induced retinal tumours of the rat and baboon^{11,12}. In Ad 12 HER1 cells we also observed oval membranous structures adjacent to the cilia (Fig. 4b). Although we have been unable to identify these bodies with certainty, they are reminiscent of the outer segments of photoreceptor cells; similar disk-like membranous structures have been found in human retinoblastoma cells¹⁹.

Ad 12 HER1 cells are completely permissive for Ad 12 virus replication and we are attempting to isolate Ad 12 E1 mutants by complementation on this cell line. However, recombination between transfecting mutated viral genomes and the abundant Ad 12 sequences in this cell line, may preclude the isolation of E1 mutants. Therefore, we are also attempting to isolate mutants on other pAsc2-transformed HER and HEK lines which contain low copy numbers of integrated Ad 12 early region 1.

Whereas the use of liquid-phase saturation-hybridization with radiolabelled adenovirus DNA to screen DNA and RNA from a large number of human cancers failed to detect adenovirus-specific sequences^{20–23}, it has been claimed that adenovirus-related mRNA can be detected by *in situ* hybridization of radiolabelled adenovirus DNA onto cryostat sections of human neurogenic tumours²⁴. Although many adenovirus serotypes cause eye infections, the only circumstantial evidence that Ad 12 may cause ocular diseases is derived from animal models of retinoblastoma^{11,12}. The evidence presented here demonstrates that cloned Ad 12 E1 region can transform human cells, and as Ad 12-transformed human embryo retinoblasts produce tumours which resemble poorly differentiated retinoblastomas, we believe that further study of retinoblastomas for adenovirus nucleic acids is warranted.

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Circulating immune complexes in infants fed on cow's milk

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Delire *et al.*¹ have reported antigen-antibody (Ag-Ab) complexes in newborns, fed on cow's milk, 6 days after birth, whereas they were absent from breast-fed neonates. The method they used was the inhibition of latex-IgG agglutination by polyclonal rheumatoid factor (pRF), linked to the Ag-Ab complexes². The presence of immune complexes was attributed to the passage through the placenta of maternal IgG with antibody activity against proteins in cow's milk. This interpretation was also based on the characterization of the Ag-Ab nature of the immune complex-like material. We have now used the same and a different method to study a similar population of newborns. Our results are very different from those of Delire *et al.* We conclude that the possible pathogenicity of immune complexes cannot be assessed on the basis of a single insensitive test and in the absence of symptoms of serum sickness.

We have studied a population of newborns similar to that examined by Delire *et al.*, 24 bottle-fed and 53 breast-fed, for the presence of immune complexes in cord sera and serum samples collected 6 days after birth. We have used the same method as Delire *et al.*¹ and, in addition, the Clq-SP assay of Hay *et al.*³.

Our results obtained with the pFR-inhibition test were very different from those of Delire *et al.*; in fact we observed a very

ing activity) and on the IgG levels on the surface of the latex particles. Furthermore, it is very important that the presence of immune complexes be linked with symptoms of serum sickness.

Thus, we believe that it is impossible to draw conclusions about the pathogenic role of Ag-Ab complexes on the basis of a single insensitive test and in the absence of characteristic symptoms.

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Binding of C3b proceeds by a transesterification reaction at the thiolester site

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The binding of C3b, the opsonic fragment of the third component of complement (C3), to bacterial surface structures mediates two events important in host defence: assembly of the C5b-C9 lytic complex and opsonic recognition by phagocytic cells (for reviews see refs 1, 2). These interactions proceed through a labile binding site³ located on the α' chain of the C3b molecule, with the resultant formation of a covalent oxy-ester bond^{4,5} in which the acyl group is contributed by the protein. By exposure of a titratable sulphhydryl group^{6,7} an internal thiolester of native C3 has also been localized to the α' chain. Studies with ¹⁴C-methylamine, a nucleophile which is inherently reactive with a thiolester, have further indicated a stoichiometric (1:1) and covalent interaction, again within the α' chain⁸⁻¹⁰. After reaction of the native protein with ¹⁴C-methylamine and radioalkylation of the exposed sulphhydryl with ³H-iodoacetic acid, a 35-residue tryptic peptide has been isolated that yields the sequence -Cys⁹-Gly-Glu-Glu¹²- on Edman degradation; tritium counts are released at step 9 (S[³H]-(carboxymethyl)cysteine) and ¹⁴C counts at step 12 (γ -glutamyl[¹⁴C]methylamide)⁸. We now present data which directly demonstrate that the second glutamyl residue of the reactive thiolester can, on proteolytic cleavage of the protein, donate its carbonyl group in a transesterification reaction with appropriate acceptor molecules. These results provide a model for analysis of the interactions at the molecular level between surface constituents of microorganisms and C3b.

Small molecules such as oligosaccharides and amines have been shown to inhibit C3 uptake onto Sepharose-trypsin matrices^{11,12}, while other studies have shown direct incorporation of radiolabelled sugars and amino acids into nascent C3b and have localized the site of incorporation to the α' chain^{13,14}. To define this site more precisely, purified human C3¹⁵ was cleaved into fragments C3a and C3b in the fluid phase by limited tryptic digestion¹⁶ in the presence of 100 mM ¹⁴C-sucrose, ¹⁴C-D-glucosamine or ¹⁴C-glycerol (NEN; each diluted to a specific activity of 1.5 mCi mmol⁻¹). For all experiments, C3 was >80% native as assessed by sulphhydryl group titration in the presence and absence of methylamine^{8,16}. Controls for each reaction included native C3 without trypsin (C3) and pretrypsinized C3 (C3b), incubated in identical conditions of temperature

Table 1 Immune complexes detected by pRF-inhibition method in neonates

Subjects	N	Cord blood	Day 6
Breast-fed	53	20 (38%)	19 (36%)
Bottle-fed	24	5 (21%)	4 (17%)

high incidence of immune complexes in umbilical cord sera, and no significant change 6 days after birth. Furthermore, there was no difference between breast-fed and bottle-fed neonates and the increase in the number of immune complexes was absolutely random (Table 1).

In contrast, using the Clq-SP assay we found only three samples of cord sera positive for immune complexes and, 6 days after birth, only two sera were positive, both in the breast-fed group (Table 2).

These observations stress the fact that the same serum populations can give very different results with different methods for immune complex evaluation. In the Clq-SP assay, using

Table 2 Immune complexes detected by Clq-SP method in neonates

Subjects	N	Cord blood	Day 6
Breast-fed	53	3 (6%)	2 (4%)
Bottle-fed	24	0	0

Staphylococcus aureus protein A as detector, it is mainly IgG1 complexes that are identified, whereas the pRF-inhibition test has a wider spectrum of detection.

The difference between our data and those of Delire could be due to the fact that the pFR-inhibition test used, although simple and easy to perform, has too subjective a reading, depends on the source of pFR used (which may affect agglutinat-

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Table 1 Incorporation (mol per mol) of radioactive ligand into total C3

	C3	C3b	C3 + trypsin
^{14}C -sucrose	0.42	0.45	0.72
^{14}C -glucosamine	0.19	0.13	0.20
^{14}C -glycerol	0.04	0.04	0.35
^{14}C -threonine	0.02	0.05	0.63

Incorporation was determined by measurements of radioactivity and absorbance at 280 nm ($E_{1\text{ cm}}^{1\%} = 9.3$)²⁴.

(37 °C) and time with each of the radiolabelled compounds. Results of SDS-polyacrylamide gel electrophoresis and autoradiography are presented in Fig. 1. For each sample, incorporation of ligand, expressed as mols of ligand per mol C3, is shown in Table 1. An identical study was performed with 100 mM ^{14}C -L-threonine (Amersham; uniformly labelled, diluted to a specific activity of 0.83 mCi mmol⁻¹), and the results are shown in Fig. 2 and Table 1.

These experiments demonstrate that generation of C3b (C3 + trypsin) in the presence of either ^{14}C -glycerol (Fig. 1) or ^{14}C -threonine (Fig. 2) results in covalent binding of the ligand

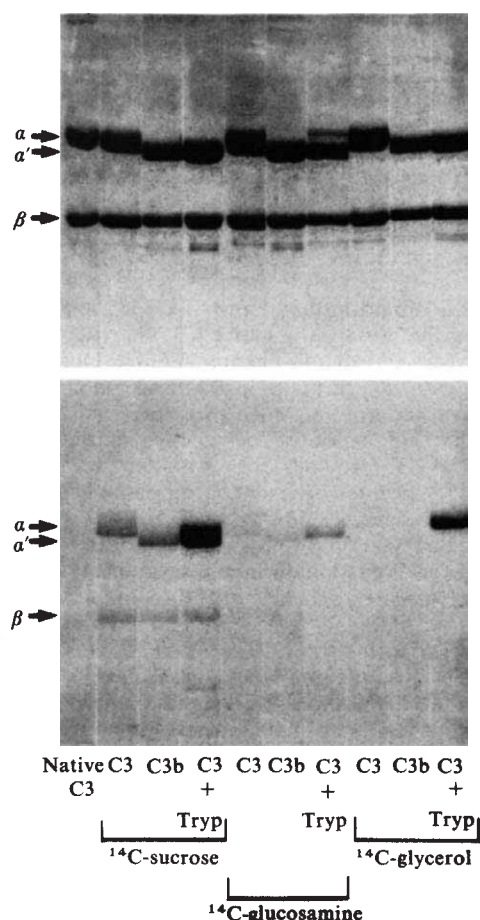


Fig. 1 SDS gel and autoradiogram of human C3 preparations reacted with ^{14}C -sucrose, ^{14}C -D-glucosamine and ^{14}C -glycerol. Each sugar derivative was reacted with the following C3 species: native (C3); pretrypsinized (C3b); trypsin-activated (C3 + trypsin). The far left lane represents untreated native C3. Purified C3 was dialysed overnight at 4 °C against 0.01 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.002 M Na₂EDTA. Trypsin digests (0.05%, w/w) were terminated at 16 min (37 °C) with the addition of a 10-fold molar excess of soybean trypsin inhibitor (Worthington). Samples were reduced in 1% SDS, 0.01 M dithiothreitol (2 h, 37 °C), alkylated with iodoacetamide (10% in excess of reducing thiol) and dialysed against 0.1 M Tris-HCl (pH 8.0), 0.01 M Na₂EDTA, 0.1% SDS. In the upper panel the chain structure of C3 (α , β) and C3b (α' , β) is visualized by staining the 7.5% polyacrylamide slab gel²⁵ with Coomassie blue R250. The lower panel shows the corresponding gel prepared for autoradiography²⁶.

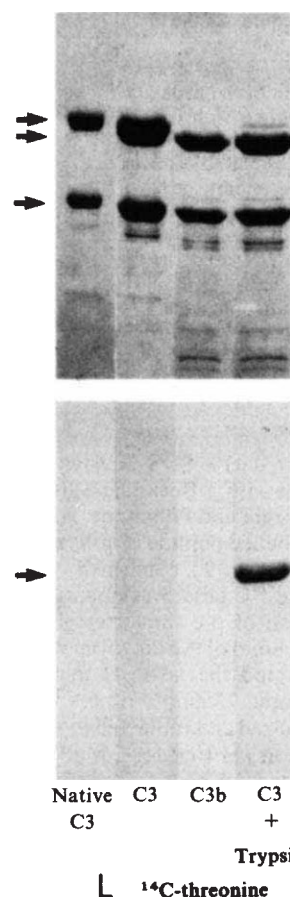


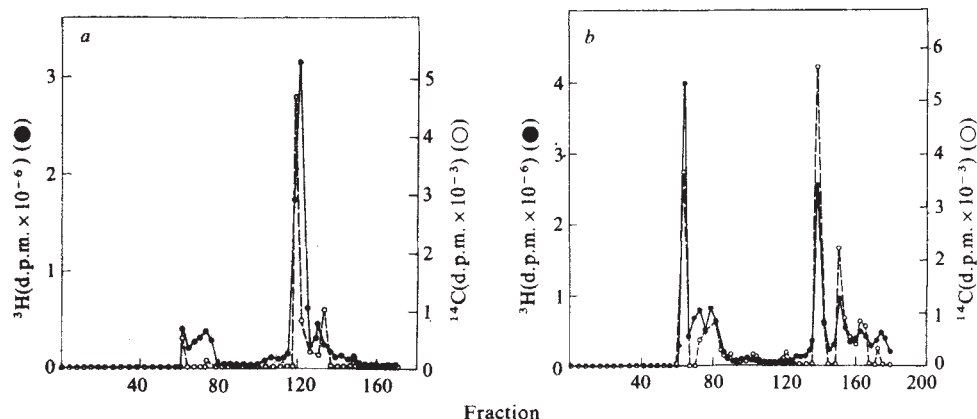
Fig. 2 SDS gel (upper panel) and autoradiogram (lower panel) of human C3 preparations reacted with ^{14}C -L-threonine. See Fig. 1 for description of method.

specifically to the α' chain. The negligible (≤ 0.05 mol per mol) incorporation of these two compounds into the control samples (C3, C3b) confirms that proteolytic cleavage of native C3 is a requirement for the covalent binding interaction and emphasizes the short lifetime of the labile binding site in C3b. In contrast, although ^{14}C -sucrose and ^{14}C -glucosamine are incorporated preferentially into the α' chain (C3 + trypsin), a significant amount of label is associated with both chains of the control C3 and C3b samples (Fig. 1). These high levels of nonspecific incorporation for sucrose and glucosamine precluded their use in further studies (Table 1). By analogy, radio-labelled glucose preparations have frequently been shown to be contaminated with high specific activity components which react nonspecifically and covalently with proteins¹⁷.

Accordingly, preparative experiments which would yield sufficient quantities of glycerol- or threonine-labelled protein for sequence analysis were performed. Purified human C3 (50 mg) was reacted with TPCK-trypsin in limited conditions in the presence of 100 mM ^{14}C -glycerol (0.25 mCi mmol⁻¹) or 100 mM ^{14}C -threonine (0.28 mCi mmol⁻¹). The mixture was treated with activated thiol-Sepharose and the bound C3b subjected to extensive trypsin digestion. Isolation of the tryptic peptide containing the labile binding site and radio-alkylation of the exposed sulphhydryl group with ^3H -iodoacetic acid (54.6 mCi mmol⁻¹) were carried out as previously described⁸. On elution of the tryptic peptides from Sephadex G-75 (Superfine), ^3H and ^{14}C radioactivity (d.p.m.) coincided in a single peak (Fig. 3) which was subsequently subjected to Edman degradation. The mol fraction of C3 that incorporated ^{14}C -glycerol or ^{14}C -threonine was 0.35 and 0.38, respectively. For the threonine-labelled peptide, material in the excluded peak represented incompletely digested sample.

The threonine-bearing peptide was lyophilized, dissolved in 1.0 ml distilled water and dialysed against 0.001 M *N*-ethyl-

Fig. 3 Elution profile of the ^{14}C -glycerol-labelled (a) and ^{14}C -threonine-labelled (b) peptides. A 1.5×220 cm column of Sephadex G-75 SF was equilibrated with 0.01 M Tris-HCl (pH 8.0), 0.2% SDS, 0.001 M Na_2EDTA . Radioactivity coincided with the major peak of absorbance at 230 nm (not shown). The yield of glycerol-labelled peptide (35%) was calculated based on the known specific activity of ^3H -iodoacetic acid and nanomols of C3b bound to thiol-Sepharose. The yield of threonine-labelled peptide was 33%.



morpholine (pH 7.5), 0.01% SDS. Automated Edman degradation was carried out with a Beckman 890C sequencer using a 0.1 M Quadrol program and Polybrene. Analysis of 23.6 nmols of ^{14}C -threonine-labelled peptide (Fig. 4) showed the ^3H counts to be present at step 9, confirmed as S -[^3H](carboxymethyl)cysteine; the ^{14}C label was quantitatively recovered at step 12, the location of the thiolester glutamyl residue. The predicted oxy-ester bond of the polyol-modified peptide proved too labile to withstand the acid pH inherent in the Edman procedure. Therefore, 23 nmols of the ^{14}C -glycerol-bearing peptide were lyophilized and subjected to ammonolysis in 1.0 M ammonium acetate at pH 10.0 for 2 h at 37°C . An additional 22.5 nmols of the glycerol-bearing peptide were left unreacted as a control, lyophilized and then dissolved in 1.0 ml distilled water. After dialysis against 0.001 M N -ethylmorpholine (pH 7.5), 0.01% SDS, the peptide treated with ammonium acetate released 66% of its ^{14}C counts, while all ^{14}C radioactivity remained bound in the control peptide. In neither peptide was release of ^3H counts detected. On Edman degradation of the amidated peptide, the ^3H label was recovered at step 9, identified as S -[^3H](carboxymethyl)cysteine, and $>95\%$ of the predicted glutamine was detected at step 12. For the experimental peptide, the recoveries of the phenylthiohydantoin derivatives were as follows for steps 9–13: (9) Cys, 2.3 nmols; (10) Gly, 1.6 nmols; (11) Glu, 1.1 nmols; (12) Glu, 0.7 nmols and Gln, 0.4 nmols; (13) Asn, 0.8 nmols. No glutamine was detected by HPLC at any of the first 14 steps in the non-amidated peptide.

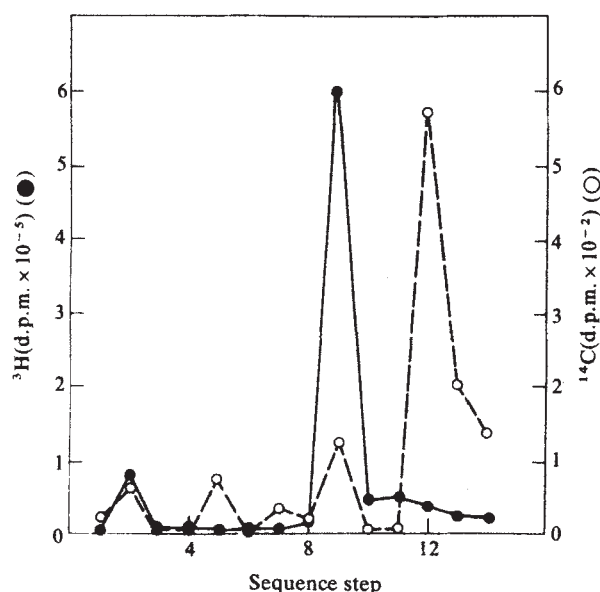


Fig. 4 Profile of the ^3H and ^{14}C counts observed on Edman degradation of the ^{14}C -threonine-labelled peptide. The recoveries of specific counts were 33% of the ^3H at step 9 and 16% of the ^{14}C at steps 12, 13 and 14.

The specific labelling data obtained in these studies clearly demonstrate that the second glutamyl residue, contained in the C3 sequence -Cys-Gly-Glu-Glu-, is the reactive species involved in covalent bonding with small molecules. In the native protein this residue participates in the formation of a thiolester bridge to the cysteinyl sulphhydryl group. On proteolytic cleavage, this carbonyl group transesterifies with an acceptor molecule, releasing the cysteinyl sulphhydryl. The reaction with glycerol is clearly one of O -acylation. For L-threonine, the resultant linkage with C3 is amide in nature (hydroxylamine insensitive¹⁴, stable on Edman degradation). It is not possible, however, to distinguish between direct N -acylation of the α -amino group and/or O -acylation at the β -hydroxyl group with subsequent rearrangement through an O - N shift. N -acylation has been proposed to account for a stable linkage observed between C3b and the Fd region of rabbit IgG¹⁸.

Recently, the thiolester site has been positioned by sequence analysis at the amino-terminal end of C3d¹⁹, a subdomain of the α' chain. The presence in native C3 of a reactive thiolester is not, however, an isolated phenomenon: evidence has been presented for an identical site in α_2 -macroglobulin^{8,20} and in C4^{21,22}—two other proteins which also function in host defence. Thus, the characterization of the reactive species involved in the binding of C3b provides both a model and a method for identifying specific sites of acylation on pathogenic organisms in which phagocytosis is mediated by C3b.

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Translation of mRNA for human granulocyte-macrophage colony stimulating factor

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Granulocyte-macrophage colony stimulating factors (GM-CSFs) regulate the growth and differentiation of committed granulocyte and macrophage progenitor cells^{1–3}. Operationally, the factors are recognized by their ability to stimulate the formation of granulocyte and macrophage colonies in semi-solid cultures of bone marrow cells. GM-CSFs are produced by a variety of tissues, and prominent cellular sources in man include T lymphocytes and mononuclear phagocytes^{1,4–7}. The factors obtained from most sources are glycoproteins of molecular weight 20,000–70,000. At least two major GM-CSF subtypes have been distinguished in mice and humans on the basis of biological activity *in vitro*: one primarily stimulates the formation of macrophage colonies, while the other stimulates the formation of both granulocyte and macrophage colonies^{1,8}. We report here the translation and partial characterization of a messenger RNA for human GM-CSF that stimulates both granulocyte and macrophage colonies. The mRNA was isolated from a human T-lymphocyte cell line, and when injected into *Xenopus laevis* oocytes⁹ it directed the synthesis of biologically active GM-CSF.

Human GM-CSFs have been partially purified and characterized from urine and from medium conditioned by placenta, leukocytes, lung and several tumour cell lines¹. For our studies of GM-CSF mRNA we used a human cell line (Mo) containing predominantly helper T lymphocytes that constitutively produces and secretes into the medium a GM-CSF stimulating the formation of both granulocyte and macrophage colonies *in vitro*^{10,11}. The Mo cell line is an exceptionally rich source of GM-CSF and unlike most other sources, appears to produce a single GM-CSF subtype. The production of GM-CSF is approximately doubled by the inclusion of phytohaemagglutinin in the culture medium¹⁰. Physically the Mo cell GM-CSF is an acidic glycoprotein of molecular weight ~30,000 (ref. 11).

Whole cell RNA was isolated from Mo cells using a guanidine thiocyanate extraction procedure¹², and poly (A)-containing RNA was enriched using oligo (dT)-cellulose chromatography¹³. The resultant poly(A) RNA was contaminated to ~50% with ribosomal RNA as judged by agarose gel electrophoresis and a tritiated poly(U) hybridization assay¹⁴. Oocytes were injected with 30–50 ng of the poly(A) RNA, and after 1–3 days' incubation the oocyte culture medium stimulated the formation of clusters and small colonies in agar cultures of human bone marrow. For example, when injected oocytes were incubated for 3 days in Barth's medium (20 µl medium per oocyte) con-

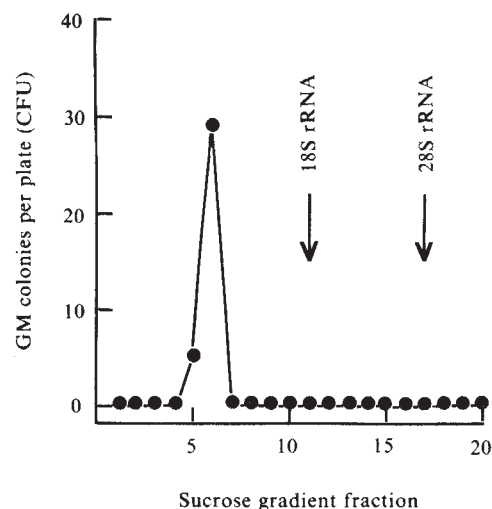


Fig. 1 Fractionation of Mo T-cell poly(A)-containing RNA using sucrose gradient centrifugation. Mo cells were grown and induced for 48 h with phytohaemagglutinin (Burroughs-Wellcome) as described previously²¹. In these conditions GM-CSF production increased approximately two-fold over that of uninduced cells¹⁰. Total RNA was isolated by a procedure involving guanidine thiocyanate extraction followed by centrifugation through a caesium chloride cushion¹². The poly(A) RNA was then isolated by chromatography on oligo(dT)-cellulose (Collaborative Research). Judging from electrophoretic analysis and a tritiated poly(U) hybridization assay, ~50% of the RNA at this stage was ribosomal¹⁴. The yield of total RNA from 10⁹ cells averaged 3–4 mg, and of this ~3% was recovered following oligo(dT)-cellulose chromatography. The RNA had a 260 nm/280 nm absorbance ratio of 2.1 or better, indicating low protein contamination. The poly(A) RNA was heated at 65 °C for 1 min and 100 µg was applied to a 10-ml 5–25% sucrose gradient containing 0.1 M sodium chloride, 10 mM Tris pH 7.4, and 1 mM EDTA. The gradient was centrifuged in a Beckman SW 41 rotor at 26,000 r.p.m. for 19 h at 4 °C and 0.5-ml fractions were collected and precipitated with two volumes of ethanol. The RNA precipitates were dissolved in 5 µl of water. An aliquot of the RNA was removed for analysis by electrophoresis on 1% agarose gels; the results indicated good separation of RNA on the basis of size, with 18S and 28S ribosomal RNA being present mainly in fractions 11 and 17. RNA from each fraction was injected into 20 oocytes (50 nl per oocyte) and the pooled oocytes from each fraction were incubated at room temperature in 200 µl of modified Barth's medium containing 1% bovine serum albumin (Sigma, crystallized) for 2 days. The medium from each fraction was then assayed for GM-CSF using cultures of human bone marrow as described in Fig. 2 legend. Fraction 6, containing the bulk of the GM-CSF mRNA activity, contained ~4 µg of RNA. The arrows indicate the positions of 18S and 28S ribosomal RNA markers present in a gradient run in parallel. CFU, colony-forming units.

taining 0.1% bovine serum albumin, 200 µl of the medium stimulated the formation of an average of ~40 small colonies containing 15 or more cells in the standard GM-CSF assay. The assay involves monitoring the formation of granulocyte and macrophage colonies in 1-ml cultures containing 10⁵ light-density human bone marrow cells plated in 0.3% agar, α-medium and 10% fetal bovine serum^{2,15,16}. In contrast, the medium from oocytes injected with water or poly(A)-containing RNA from rat liver did not stimulate colony formation.

For these experiments it was important to use non-adherent bone marrow cells in the assay because in some cases the presence of adherent cells in the cultures produced a background of clusters that made it difficult to detect the small amount of activity present in oocyte medium¹⁵. Also, the presence of protein (0.1% bovine serum albumin) in the oocyte medium appeared to stabilize the activity stimulating colony formation. Homogenates of oocytes injected with Mo poly(A) mRNA stimulated few colonies; this phenomenon may be attributed in part to an inhibitor of colony formation present in the oocytes (see below). The numbers of colonies produced in different experiments varied considerably, probably due to donor-specific differences in bone marrow and variability in frog oocytes. This variability made it difficult to ascertain whether treatment of the Mo cells with phytohaemagglutinin stimulated GM-CSF mRNA activity; nevertheless, for experi-

ments described below, RNA was extracted from Mo cells treated with the lectin.

A control experiment in which mRNA preparations were added directly to bone marrow cultures indicated that contaminating GM-CSF in the RNA was not responsible for the stimulation of colony formation. The addition of 500 ng of sucrose gradient-purified GM-CSF mRNA (see below) to duplicate cultures of bone marrow cells did not result in the stimulation of colony formation, while medium derived from 10 oocytes injected with a comparable amount of the same mRNA stimulated the formation of up to 30 granulocyte-macrophage colonies containing ≥ 20 cells.

The CSF signal obtained when total poly(A) RNA was injected into oocytes was usually low, with few colonies containing relatively small numbers of cells. The poly(A) RNA was therefore fractionated according to size using sucrose gradient centrifugation, and the resultant fractions injected into oocytes. RNA in each fraction from the gradient was precipitated with ethanol and resuspended in 5 μ l of H₂O. Agarose gel electrophoresis and *in vitro* translation using reticulocyte lysates¹⁷ demonstrated the presence of translatable poly(A) mRNA in each fraction. An aliquot (50 nl) of each fraction was injected into each of 20 oocytes, and the oocytes from each fraction were incubated together in 20 μ l of medium for 2 days. Assay of the medium in human bone marrow cultures indicated that the CSF activity was present in a single sharp peak of RNA from the sucrose gradient (Fig. 1). Using 18S and 28S ribosomal RNA markers, the size of the GM-CSF mRNA was estimated at about 1,200 nucleotides. A mRNA of this size could be translated into a protein having a maximum molecular weight of 44,000—this compares with a molecular weight of $\sim 30,000$ for GM-CSF produced by the Mo cells as estimated by gel filtration chromatography¹¹ or SDS-polyacrylamide gel electrophoresis (A.J.L., unpublished). As the Mo GM-CSF is gly-

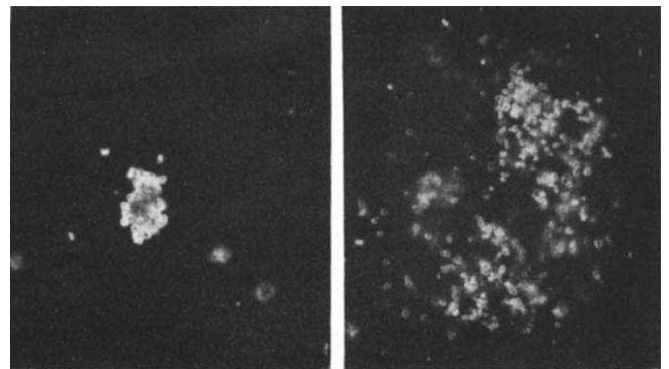


Fig. 3 Gross morphology of typical granulocyte-macrophage colonies stimulated by GM-CSF translated in frog oocytes. Both 'tight' (left) and 'loose' (right) colonies were also observed at low concentration of Mo T-cell conditioned medium (not shown). $\times 30$

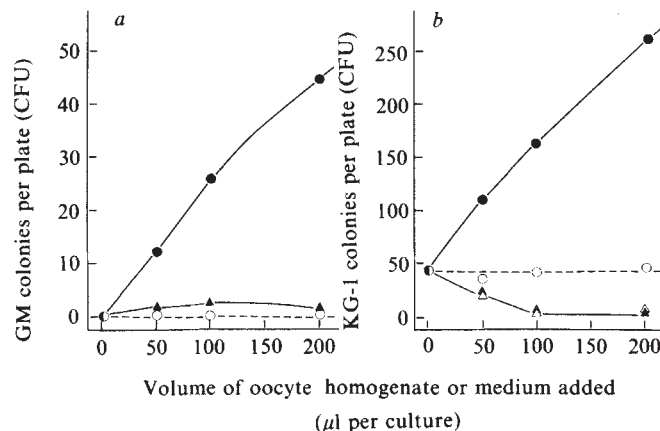


Fig. 2 Clonal assay of GM-CSF translated by oocytes using human bone marrow (a) and the KG-1 human myeloid leukaemia cell line (b). Oocytes were injected with 40 ng of RNA purified from the Mo T-cell line using oligo(dT)-cellulose chromatography and sucrose gradient centrifugation as described in Fig. 1 legend. The oocytes were incubated, in pools of 10, in 200 μ l of modified Barth's medium containing 1% bovine serum albumin for 2 days at room temperature. The oocyte incubation medium was removed and the oocytes were homogenized in 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4 (20 μ l per oocyte). The debris was removed by centrifugation at 12,000g for 10 min. The oocyte homogenates and media were then assayed in clonal assays using human bone marrow target cells as previously described^{11,15,16}. Briefly, bone marrow cells were aspirated from healthy adult volunteers, and the light-density, non-adherent cells were isolated using sedimentation in Ficoll-Hypaque followed by Petri dish adherence. 10^5 fractionated cells were plated with varying amounts of oocyte homogenates or media in 1-ml cultures containing 0.3% agar, 10% fetal bovine serum, α -medium (Flow) and α -thioglycerol. The cultures were then incubated for 11 days at 37 °C in 7% carbon dioxide, when they were examined using a low power microscope and colonies of ≥ 30 cells counted. In tests on the KG-1 human myeloid leukaemia cell line, the cells were plated in agar and cultured at a density of 2×10^5 cells m^{-2} culture exactly as described for bone marrow cells. Colonies containing ≥ 20 cells were counted after 11 days. Each point represents the average of two determinations. Medium from oocytes injected with mRNA (●) or water (○); homogenates of oocytes injected with mRNA (▲) or water (△).

cosylated, the information content in the mRNA is more that sufficient to encode this protein. The sucrose gradient fractionation was performed three separate times with identical results.

The enrichment of mRNA for GM-CSF by sucrose gradient fractionation resulted in a considerably stronger stimulation of colony formation by oocyte medium than was obtained using total poly(A)-containing RNA. In contrast to the relatively small colonies observed using unfractionated RNA, peak gradient fractions, when injected in quantities of 25–50 ng per oocyte, stimulated relatively large colonies containing up to ~ 300 cells. For example, in the experiment shown in Fig. 2, 200 μ l of oocyte medium derived from 10 oocytes injected with fractionated mRNA and incubated for 2 days, stimulated more than 40 colonies containing ≥ 30 cells as well as numerous smaller clusters. In terms of colony number, size and morphology, the GM-CSF in 200 μ l oocyte medium resulted in stimulation equivalent to ~ 4 μ l of medium conditioned by the growth of Mo cells for 3 days at a final density of 5×10^6 cells ml^{-1} (Fig. 3). Because of its higher specific activity, sucrose gradient fractionated preparations of GM-CSF mRNA were used in the experiments described below.

The GM-CSF produced by frog oocytes stimulated colony formation dose dependently (Fig. 2). The curve resembles that found previously for the GM-CSF produced by Mo T cells^{10,11}. Also, the activity in oocyte medium produced colonies that were similar morphologically to those observed using low levels of T-cell conditioned medium (Fig. 3). Microscopic examination of individual cells from the colonies indicated the presence of monocytic, granulocytic and mixed monocytic-granulocytic colonies (data not shown). For these experiments, single colonies were picked from agar cultures using a fine pipette, dissociated from the agar by a stream of air and stained with Wright's or Giemsa. Monocytic colonies were identified using a lipase stain specific for monocytes and macrophages¹¹. Using this procedure, 50–100 μ l of medium from injected oocytes stimulated an average of 56% monocytic colonies, 37% granulocytic colonies and 7% mixed colonies. A comparable concentration of T-cell-derived GM-CSF gave similar results, with 49% of the colonies being monocytic, 42% granulocytic and 9% mixed.

The bulk of the GM-CSF activity produced by the oocytes was apparently secreted into the medium (Fig. 2). However, the determination of GM-CSF activity in oocyte homogenates is complicated by the presence of a weak inhibitor of colony formation. For example, in mixing experiments, 200 μ l of control oocyte homogenate from the experiment shown in Fig. 2 produced $\sim 40\%$ inhibition of the activity of added Mo T-cell GM-CSF. Taking this inhibition into account, $\sim 90\%$ of the GM-CSF produced by oocytes is present in the medium after 2 days. Because of the relatively small amount of activity produced by injected oocytes, a detailed physical characterization of the oocyte-derived GM-CSF has not been possible. However, preliminary experiments have shown that the activity

produced by oocytes had a heat inactivation profile similar to that of the GM-CSF produced by Mo T cells. When incubated at neutral pH for 30 min both activities were stable at 60 °C but were almost completely inactivated at 80 °C.

The GM-CSF translated in oocytes also stimulated the growth in agar of a human myeloid leukaemia cell line, KG-1, that has previously been shown to be specifically responsive to GM-CSF^{18,19} (Fig. 2). As in the case of bone marrow cells, medium from oocytes injected with water or control RNA had no effect on the growth of KG-1 cells. However, homogenates of both injected and uninjected oocytes significantly inhibited cell growth (Fig. 2).

The GM-CSF mRNA assay will undoubtedly be useful for the isolation of cloned cDNAs which contain GM-CSF nucleo-

tide sequences. Similar oocyte translation systems have proved useful for studies of interferons²⁰. Clearly, molecular genetic approaches should aid the examination of the structural and functional properties of these mediators of haematopoiesis. Our results also suggest that it should be feasible to use the oocyte system for the translation and characterization of mRNAs for other factors regulating the growth and differentiation of haematopoietic cells.

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A common mechanism for the synthesis of membrane and secreted immunoglobulin α , γ and μ chains

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Immunoglobulins serve as both secreted effector antibodies and antigen receptors bound to the membrane of lymphocytes. Each newly generated B lymphocyte expresses membrane IgM and subsequently co-expresses membrane IgM and membrane IgD. Antigen-driven clonal proliferation is accompanied by further differentiation with the generation of B lymphocytes expressing, in membrane-bound form, other classes and subclasses of immunoglobulin¹. Such memory cells are apparently capable of responding to antigen to generate further clones of cells secreting antibodies of particular classes and subclasses. The mechanism by which IgM functions as both a membrane-bound and a secreted immunoglobulin has been determined for both murine and human systems²⁻⁴. The difference between the two forms of IgM is entirely at the C-terminus. The μ_s (secreted) chain has a hydrophilic peptide additional to the C μ 4 domain and the μ_m (membrane-bound) chain has a hydrophobic peptide replacing the hydrophilic C-terminal peptide of the μ_s chain²⁻⁴. The μ_s and μ_m chains are translated from distinct mRNA molecules that are generated by alternative splicing and/or termination points of transcripts from a single rearranged μ gene^{5,6}. Recent evidence supports the existence of a similar mechanism for the generation of δ_s and δ_m chains⁷⁻⁹. Structurally different γ_m and γ_s chains have been observed in human, mouse and chicken cells¹⁰⁻¹². The present study further distinguishes between γ_m and γ_s chains and characterizes the mRNA molecules encoding them in human cells. We have also characterized α_m and α_s chains in a human cell. The data reported here, together with other recent findings, suggest a common mechanism for the generation of membrane and secreted forms of α , γ and μ chains.

We have compared the biosynthesis of IgG in three different human B-cell lines (EB4, Bec-11 and Maja) with that of IgA in the human cell line Dakiki Arosros I and of IgM in the human line Bjab. The membrane and secretory heavy chains of Bec-11, Dakiki and Bjab immunoglobulins have been fully characterized previously^{10,13}. Two forms of biosynthetically labelled, specifically immunoprecipitable γ chains of EB4 cells are clearly resolved by SDS-gel electrophoresis (Fig. 1), differing in apparent molecular weight by 12,000 whether glycosylated or not. The high molecular weight band can be identified as γ_m by iodination of intact cells (Fig. 1e), and the lower molecular weight γ chain as γ_s by demonstrating its secretion in either the glycosylated (Fig. 1h) or non-glycosylated (lane i) state. The cell line Maja synthesizes two γ polypeptide chains similar in size to the γ_m and γ_s chains of Bec-11 and EB4 cells. M. J. Owen (personal communication) has characterized the γ_m and γ_s chains of Maja cells. The three cell lines show three different patterns for glycosylation of γ chains: in EB4, both γ_m and γ_s seem to be singly glycosylated; in Bec-11 some γ_s chains have a second oligosaccharide unit added but γ_m chains are singly glycosylated; and in Maja cells a substantial proportion of both γ_m and γ_s chains have a second oligosaccharide unit added to the polypeptide chain.

When non-glycosylated, the γ chains of the three cell lines have different electrophoretic mobilities but in each line the apparent molecular weights of the γ_m and γ_s forms differ by ~12,000. This is in contrast to the difference in apparent

Table 1 C-terminal amino acid sequences of human immunoglobulin heavy chains

Protein*	H chain class	Residue no.	Sequence
Ou (16)	μ	609	GKPTLYNVSLVMSDTAGTCY
Bur (17)	α	477	GKPTHVNVSVVMAGVDGTCY
Zuc (18)	γ	477	G (K)
ND (19)	ϵ	609	G K

The C-terminal amino acid sequences of human μ , α , γ and ϵ chains from the glycine residue located at the end of the last constant region domain to the end of the polypeptide are shown. The myeloma proteins given are representative of the equivalent sequences found in other myelomas. The C-terminal lysine of the γ -chain is shown in parentheses as it is not always found in the polypeptide although it is ubiquitous in the gene sequence¹⁵.

* References given in parentheses.

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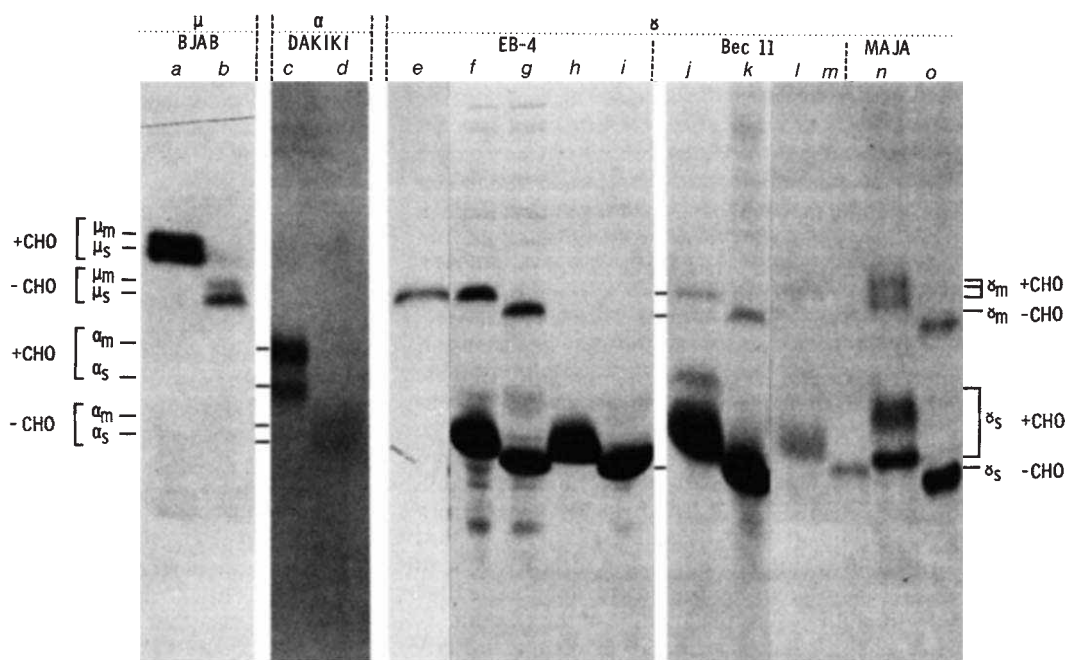
molecular weight between μ_m and μ_s (~3,000) and α_m and α_s (~2,000). Comparison of the C-terminal amino acid sequences of α_s , γ_s and μ_s chains reveals a conserved Gly-Lys sequence at the end of the terminal domain followed, in the case of α and μ chains, by highly homologous hydrophilic tail sequences (Table 1). Neither ϵ nor γ_s chains possess this tail sequence (Table 1). In the murine μ gene, the Gly-Lys sequence encodes a consensus splice site used in the generation of μ_m mRNA³. It is therefore proposed that this splice site is conserved in α and μ genes. The similarity between the hydrophilic tail sequences of α and μ genes would be consistent with a common evolutionary origin for the membrane exons for these two classes and this would explain the similar size difference between the membrane and secreted forms of the chains. In this model, the hydrophilic tailpiece of γ_s is a single lysine residue. We propose that the hydrophobic, immunoglobulin domain-sized tailpiece on γ_m chains had a separate evolutionary origin from the M exons of α and μ chains.

To test the hypothesis that the γ_m and γ_s polypeptides are translated from different mRNA molecules, the specific mRNAs coding for γ chains in cell lines EB4 and Bec-11 were identified. Total poly(A)-containing RNA was prepared from each cell line, resolved electrophoretically in denaturing conditions, transferred to diazobenzyloxymethyl paper and hybridized to a genomic $\gamma 2$ DNA probe. This probe detects 3.3 and 1.4 kilobase (kb) γ mRNA in EB4 RNA (Fig. 2). The higher molecular weight mRNA is of lower intensity than the lower molecular weight RNA. This difference is consistent with our

interpretation that the 3.3 kb mRNA encodes γ_m and the 1.4 kb species γ_s . In the highly stringent conditions used to obtain the data shown in Fig. 2, the $\gamma 2$ probe only detects the 1.4 kb component in Bec-11 mRNA. In less stringent conditions, the $\gamma 2$ probe gives a more intense hybridization signal in the 1.4 kb position and shows some hybridization to the 3.3 kb component (data not shown). The difference between the results obtained with EB4 and Bec-11 is consistent with the much lower ratio of γ_m to γ_s polypeptides in Bec-11 cells than in EB4 cells (Fig. 1) and might be related to a subclass difference between EB4 and Bec-11 γ chains.

To account for the molecular weight difference between γ_s and γ_m polypeptide chains the γ_m message would need to be only 0.3 kb larger than the γ_s mRNA. The observed size difference of 1.9 kb is in close agreement with the reported difference of 2 kb between murine $\gamma^2 a_s$ and $\gamma^2 a_m$ mRNAs¹⁴. The most likely explanation for the unexpected size difference between γ_m and γ_s mRNAs is the presence of an extended 3'-untranslated region in the γ_m message (we have found no evidence for any other coding region in the 3.3 kb mRNA as we can detect no γ chains larger than γ_m). For comparison, μ_m and μ_s mRNA molecules are 2.7 and 2.4 kb respectively. The predicted coding difference would account for 63 base pairs (bp) and gene sequencing shows that the 3'-untranslated region of μ_m is 142 bp longer than that of μ_s mRNA³. The close agreement between the human and murine γ chain data are consistent with an early evolutionary divergence or separate origin for the M exons of γ and μ chains.

Fig. 1 Biosynthetic characterization of membrane and secretory forms of α , γ and μ polypeptide chains. The following human B-lymphoid cell lines were used: Bjab (IgM κ), Dakiki Arosros I (IgA λ), EB4 (IgG κ), Bec-11 (IgG κ), and Maja (IgG κ). An aliquot (2.5×10^6 cells) from log-phase cultures of each cell line was incubated in the presence or absence of tunicamycin ($2 \mu\text{g ml}^{-1}$) for 3 h at 37°C in RPMI-1640 medium plus 10% (v/v) fetal calf serum (FCS). After preincubation, the cells were washed once in serum-free RPMI-1640 lacking methionine and then resuspended in 0.2 ml of the same medium containing $200 \mu\text{Ci } ^{35}\text{S}$ -methionine and incubated at 38°C for 90 min to effect metabolic labelling of cellular proteins. For pulse-chase experiments (lanes h, i), 50% of the culture was pelleted, resuspended in 0.5 ml of RPMI-1640 medium containing 10% (v/v) FCS and incubated at 37°C for 4–6 h: the cells were pelleted and the supernate used for analysis of secreted proteins. Membrane proteins were radiolabelled by lactoperoxidase-catalysed iodination. 10^7 cells were incubated for 3–4 min at 30°C in $70 \mu\text{l}$ of PBS-1 (50 mM sodium phosphate buffer pH 7.2; 150 mM NaCl) containing $10 \mu\text{g}$ lactoperoxidase and $1.25 \text{ mM H}_2\text{O}_2$; the reaction was terminated by addition of ice-cold 5 mM KI in PBS-1 (1 ml). The cells were washed extensively before preparation of cell lysates. Cell lysates were prepared as follows²⁰. Radiolabelled cells were washed once in ice-cold TKM buffer (0.1 M Tris-HCl pH 8.2, 0.1 M KCl and 5 mM MgCl_2) and resuspended in $235 \mu\text{l}$ of ice-cold 1% (w/v) Triton X-100 in TKM; the suspensions were placed on ice for 20–30 min to complete lysis. Nuclei were removed by centrifugation (5,000 g for 30 min) and deoxycholate and SDS were added to the supernatant to final concentrations of 1% (w/v) and 0.5% (w/v) respectively to yield lysates in 3D-TKM (1% (w/v) Triton X-100, 1% (w/v) deoxycholate and 0.5% (w/v) SDS in TKM buffer). Radiolabelled immunoglobulin was isolated by specific immunoprecipitation using $1 \mu\text{l}$ of rabbit antiserum to the appropriate light chain followed by addition of serological equivalence of goat anti-rabbit immunoglobulin antiserum. After overnight incubation at 4°C , the immunoprecipitates were collected by centrifugation and washed by pelleting through a two-step discontinuous sucrose gradient in 3D-TKM. Aliquots of washed immunoprecipitates were dissolved in $25 \mu\text{l}$ of SDS polyacrylamide gel electrophoresis loading buffer [0.065 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1 M dithiothreitol, 10% (w/v) glycerol and 0.001% (w/v) Bromophenol blue] and heated at 100°C for 2 min. Reduced samples were electrophoresed on 10% (w/v) acrylamide slab gels using the discontinuous buffering system of Laemmli²¹ [IgM samples derived from Bjab cells were analysed on a 12.5% (w/v) acrylamide slab gel]. Radioactive components were visualized by fluorography²². Lanes a, b , glycosylated and non-glycosylated μ chains respectively; c, d , equivalent samples derived from the α -chain-producing line Dakiki Arosros I. Radioiodinated membrane γ -chain of EB4 cells is shown in lane e and is compared with glycosylated and non-glycosylated γ -chains for EB-4 lysates (f, g) and supernatants (h, i); j, k , respectively glycosylated and non-glycosylated γ chains from Bec-11 cell lysates; l, m , similar results for Bec-11 cells (obtained in an independent experiment) compared with glycosylated (n) and non-glycosylated (o) γ chains of the cell line Maja to illustrate the ubiquity of the large size difference between γ_m and γ_s polypeptides.



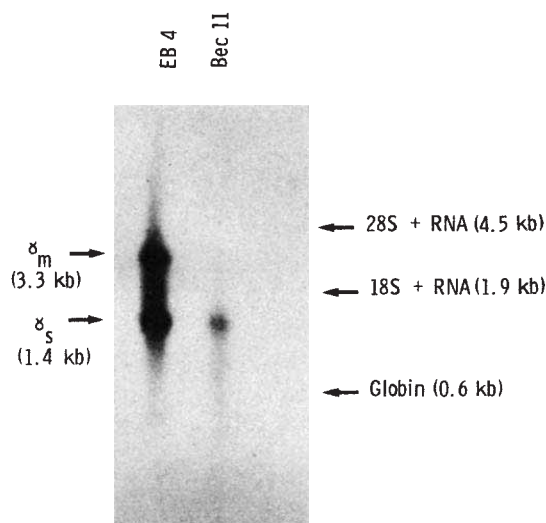


Fig. 2 Polyribosomes were prepared from 5×10^9 EB4 cells as previously described²⁰. Poly(A)-containing RNA was extracted from polyribosomes by oligo(dT)-cellulose chromatography. Five μg of EB4 poly(A)-containing RNA was electrophoresed on a 1% (w/v) agarose gel containing 7.5 mM methyl mercury hydroxide. After electrophoresis, the separated RNA species were transferred to diazobenzyloxymethyl paper²³. A DNA plasmid containing $\gamma 2$ coding sequences of human genomic DNA was nick-translated to a specific activity of 1×10^8 d.p.m. μg^{-1} with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and used as a probe for γ mRNA species contained in the EB4 poly(A)-containing RNA. Hybridization was done at 42°C for 5 h in 50% formamide, $5 \times \text{SSC}$, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll (molecular weight 400,000) 20 mM sodium phosphate pH 6.5, 10% sodium dextran sulphate 500 and $100 \mu\text{g ml}^{-1}$ denatured sonicated salmon sperm DNA. Unhybridized label was removed by extensive washing in $0.3 \times \text{SSC}/0.1\%$ SDS and hybridization visualized by autoradiography using an intensifier screen at -70°C .

In cloned murine genomic DNA, two homologous regions, 270 and 250 bp long, are conserved at a position 1–2 kb 3' to each of the γ subclass genes¹⁵. These regions are known to hybridize to the γ_m mRNA^{14,15}. They are quite sufficient to encode the additional domain-size segment of γ_m chains but would not account for the size of the γ_m mRNA. The implication that the additional γ_m mRNA sequences are not conserved between γ subclasses further supports the argument that these additional sequences are non-coding.

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Production of colony-stimulating factors by murine T cells in limiting dilution and long-term cultures

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Colony-stimulating factors (CSF) are glycoproteins essential for the formation of colonies of mature haematopoietic cells by single immature progenitor cells (colony-forming cells (CFC)). The number of colonies in semi-solid agar cultures can be used to determine the CSF concentration in biological fluids¹. Various murine cells^{2–6} are able to produce CSF *in vitro*. Supernatants of spleen cell cultures consisting of a mixture of T cells, B cells and macrophages are found to contain neutrophil-granulocyte-macrophage CSF (GM-CSF), CSF for megakaryocytes (Meg-CSF), eosinophilic granulocytes (Eo-CSF) and erythroid cells (E-CSF)⁵. The producer cells of these different CSF types, however, have not been defined⁷. We therefore determined here whether normal T cells produce CSF *in vitro* and whether GM-CSF, Meg-CSF, Eo-CSF and E-CSF are produced by different T-cell subsets or the progeny of single T-cell clones. Our results indicate that T cells at a high frequency (one out of three) produce CSF at variable amounts even in the absence of accessory cells following mitogen stimulation.

After stimulation of C57BL/6 (B6) spleen cells in bulk cultures with $5 \mu\text{g ml}^{-1}$ concanavalin A (Con A) for 48 h, light density blasts were separated by centrifugation over Ficoll (1.077 g cm^{-3}). These cells ($>95\%$ Thy-1.2 positive) were subsequently grown in microcultures in limiting dilution conditions in the presence of allogeneic (AKR/J) irradiated (3300 r ; ^{60}Co) peritoneal exudate filler cells (PEC) (10^4 per well) and 10% T-cell growth factor (TCGF) containing conditioned medium from 24 h Con A-stimulated rat (Sprague–Dawley) spleen cells (rat spleen cell conditioned medium (RSCM)). The T cells have a doubling time of ~ 15 h and clones derived from single or a few precursor cells grow to a clone size large enough to be tested in functional assays^{8–12}. Following a growth period of 10 days during which the cells were fed twice with fresh RSCM, the cells were washed twice to remove CSF contained in RSCM and resuspended in fresh culture medium containing $10 \mu\text{g ml}^{-1}$ Con A. After overnight incubation supernatants of all culture wells were assayed individually for CSF at a final concentration of 15% in 1 ml semi-solid agar cultures containing 7.5×10^4 CBA bone marrow cells. Seven days later the bone marrow cultures were scored for colony growth and the cellular composition of the colonies determined¹³.

Table 1 % CSF-negative cultures of T cells plated in the presence of different numbers of filler cells

No. of PEC per well	1 per well	T cells plated 10 per well	20 per well
5×10^2	100	100	63
10^3	100	88	75
10^4	100	19	13
10^5	81	75	69

Limiting dilution analysis of B6 splenic T cells was performed as described in Fig. 1 legend.

Table 2 Con A-induced CSF production by TCGF-dependent cells

Long-term cultured cells	Growth dependent on Antigen TCGF		Production of CSF				
			Total no. of colonies ($\pm$ s.e.)	Colonies (%)			
				GM	Meg	Eo	E
PC-AKR-clone 29	—	+	78 $\pm$ 9	97	3	0	0
AKR-anti-B6 line PK 7.1.1b	+	+	126 $\pm$ 15	86	2	6	6
AKR-anti-B6 line PK 7.1.2	+	+	113 $\pm$ 11	90	4	1	5

The establishment of PC AKR clone 29 in long-term culture from antigen (picryl chloride)-stimulated AKR/J T cells has been described previously^{18,19}, as has the establishment of the alloreactive AKR anti-B6 T-cell lines PK 7.1.1b and PK 7.1.2^{9,20,21}. Antigen used was irradiated allogeneic B6 stimulator cells. The number of colonies (controls) with a standard CSF: 127 $\pm$ 24, NaCl: 0 $\pm$ 0.

A representative experiment (Fig. 1) shows that, analysed according to the Poisson distribution (least square fit as well as the method of Porter and Berry¹⁴), the growth frequency of Con A-activated T cells initially plated in limiting dilution was approximately one out of two to three cells ($f = 1/2.2$ (least square method), $f = 1/2.5$ ref. 14, 95% confidence limit $f = 1/2.1$ to $f = 1/2.9$ (ref. 14)). The frequency of CSF producer cells was close to $f = 1/6$ (Fig. 1). Lower frequencies found in other experiments (Table 1) were essentially due to lower plating efficiencies. The plot of our data (Fig. 1) gave a straight linear regression line ($\chi^2 \sim 1$ for fit¹⁴) with an intercept at the ordinate at or close to 1.0. This suggests single-hit kinetics, indicating that the only limiting component for CSF production was the number of T cells initially seeded in microculture. This is supported by our observation that no CSF production occurred in cultures devoid of proliferating T cells (data not shown). Moreover, CSF production was not constitutive but absolutely dependent on Con A stimulation (data not shown).

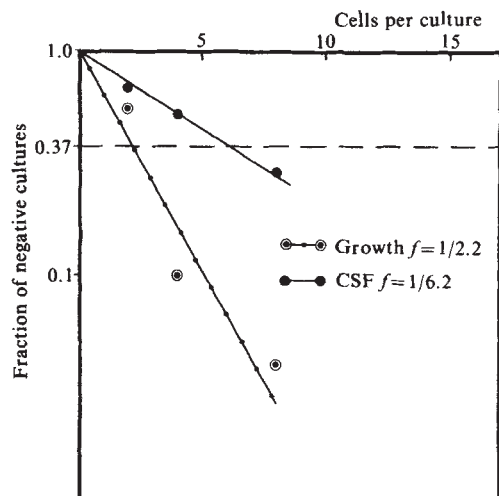


Fig. 1 Frequency of growing and CSF-producing Con A-activated splenic B6 T cells in limiting dilution. Two days after polyclonal activation by Con A and separation of the light density T-cell blasts over Ficoll, 2, 4 and 8 B6 cells were plated in microcultures (96 wells per group) and expanded on irradiated (3300 r) AKR/J PEC (10^4 per well) for 10 days in the presence of medium containing 10% RSCM from 24 h Con A-stimulated rat (Sprague-Dawley) spleen cells. After a growth period of 10 days the cells were washed twice to remove CSF contained in RSCM and resuspended in fresh culture medium containing $10 \mu\text{g ml}^{-1}$ Con A. After overnight incubation supernatants of all culture wells were assayed individually for CSF at a final concentration of 15% in 1 ml semi-solid agar cultures containing 75,000 CBA bone marrow cells. After 7 days the bone marrow cultures were scored for colony growth¹³. Cell growth in limiting dilution microwells was determined by microscopic inspection. Data were plotted according to Poisson's distribution and linear regression lines fitted to the least squares method. Frequencies were read from the number of responding cells corresponding to 37% negative cultures⁸⁻¹².

Note that the above procedure might favour selection of certain T-cell subpopulations. Con A activation (20–50% growth of Con A-activated murine spleen cells in bulk culture (data not shown)), Ficoll purification, growth in limiting dilution microcultures (growth frequency as high as $f = 1/2$) and induction of lymphokine release by Con A may have a selective effect. Nevertheless, we believe that this experimental system probably provides the most random evaluation available of the repertoire of functional T cells in non-immune mice¹².

Figure 2a shows the results of an analysis of CSF activity of supernatants from microcultures derived from the progeny of single or a few T-cell precursors. The quantity of CSF in such supernatants varied remarkably, and occasionally very high

Table 3 Specificity of induction of proliferation and CSF release

Stimulator cells	<i>H</i> -2 regions								³ H-thymidine incorporation ($\pm$ s.e.)	No. of colonies ($\pm$ s.e.)
	<i>K</i>	<i>A</i>	<i>J</i>	<i>E</i>	<i>C</i>	<i>S</i>	<i>G</i>	<i>D</i>		
AKR	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	597 $\pm$ 389	0
B6	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	25,089 $\pm$ 3,545	18 $\pm$ 5
B10.D2	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	734 $\pm$ 474	0
B10.A(4R)	<i>k</i>	<i>k</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	1,968 $\pm$ 299	0
B10.A(5R)	<i>b</i>	<i>b</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	31,263 $\pm$ 2,596	17 $\pm$ 2

10^4 Responder cells of the AKR anti-B6 T-cell line PK7.1.2 were used per well (triplicates, round-bottom microtitre plates, Greiner, Nürtingen, 96K). Irradiated (3300r) stimulator cells at a concentration of 10^5 per well were used after lysis of red blood cells. ³H-thymidine incorporation (2.5 μCi per well) was determined after a 6-h pulse on day 5 of culture. CSF was induced by overnight stimulation with stimulator cells on days 4–5 of culture. The number of colonies (controls) with a standard CSF: 78 $\pm$ 7, and NaCl: 0 $\pm$ 0. Underlining indicates the presence of the *b* allele in the subregions of the *H*-2 complex.

activities from low numbers of T cells could be observed. All active supernatants contained GM-CSF. Moreover, many supernatants with intermediate to high GM-CSF levels contained other types of CSF, for example, Meg-CSF, Eo-CSF and E-CSF. As the incidence of CFC for megakaryocytes, eosinophils and erythroid cells in the bone marrow is low, the semi-solid agar culture system only detects high concentrations of Meg-CSF, Eo-CSF and E-CSF. Therefore, some supernatants with only low GM-CSF levels may not be entirely negative for the other CSF types.

Figure 2 also shows that Meg-CSF and/or Eo-CSF and E-CSF were only found in supernatants which also contain GM-CSF. Possibly the colony-forming potential of CFC for megakaryocytes, eosinophils and erythroid cells is only expressed when stimulated by specific CSF and GM-CSF. This suggests that some supernatants might exist which only contain Meg-CSF and/or Eo-CSF and E-CSF but no GM-CSF and is compatible with data by Metcalf *et al.*¹⁵. Such supernatants would not be detectable in our standard assay. To investigate this we determined the CSF activity of one aliquot of a large number of supernatants from limiting dilution microcultures separately from another aliquot mixed with a standard CSF preparation from mouse lung conditioned medium (MLCM). This mixing experiment, however, gave no indication of the

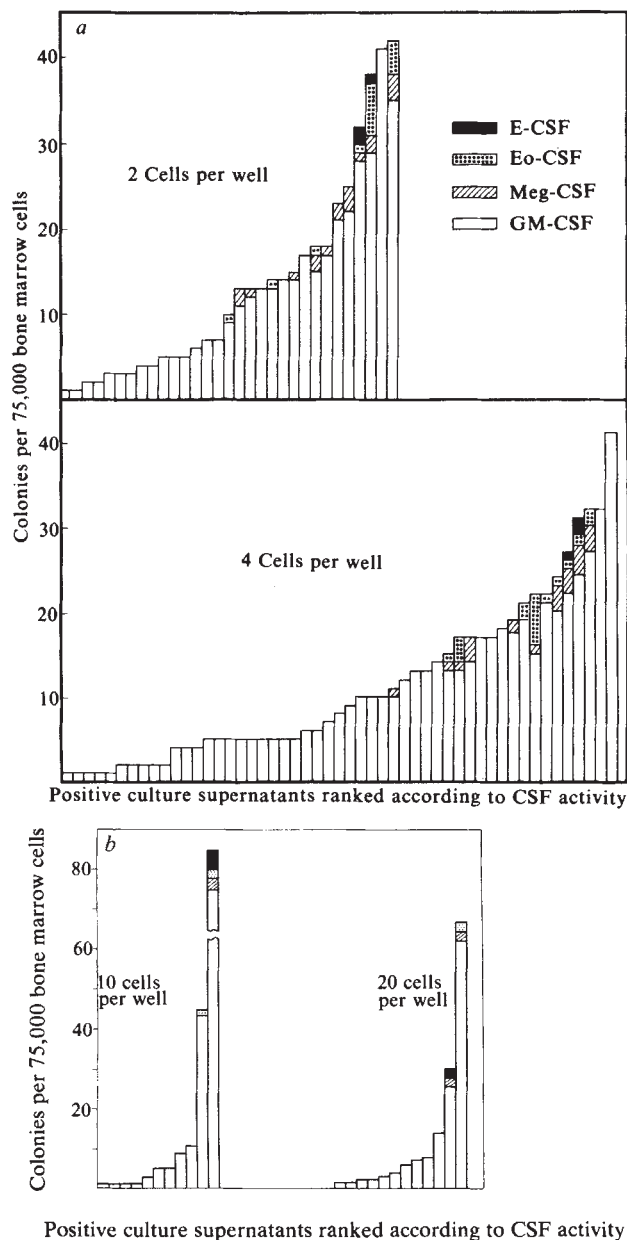


Fig. 2 *a*, Analysis of CSF types in CSF-positive culture supernatants. B6 splenic T cells were established in limiting dilution microcultures as described in Fig. 1 at an initial plating of two (upper) or four (lower) cells per well. The CSF-positive culture supernatants were ranked according to their activity. Number of colonies (controls) with a CSF standard: 84 ± 5 , and NaCl: 0 ± 0 . *b*, Analysis of CSF types in supernatants of T cells grown in limiting dilution microcultures in the presence of SRBC as filler cells. B6 splenic T cells were grown as described in Fig. 1 legend except that instead of irradiated allogeneic peritoneal exudate filler cells 10^6 SRBC from a selected batch particularly suitable for growth promotion were used as filler cells. Number of colonies (controls) with a CSF standard: 110 ± 15 , and NaCl: 0 ± 0 .

existence of supernatants containing only Meg-CSF and/or Eo-CSF and E-CSF, and the existence of potentiators or inhibitors of standard CSF in these supernatants could not be demonstrated (data not shown).

The above experiments suggest that different CSF types are produced by T cells. As T cells were plated in limiting dilution microcultures in the presence of allogeneic irradiated PEC it had to be excluded, however, that these PEC were involved in CSF production. Table 1 shows that no linear correlation could be found between the number of CSF-positive wells and the number of irradiated PEC used in the microculture system. In fact, at the highest number of PEC (10^5 per well) a decrease

rather than an increase of CSF-positive culture supernatants occurred. In addition, when selected batches of sheep red blood cells (SRBC) instead of PEC were used as filler cells CSF production was still detectable. Although the growth frequency of T cells in these cultures was comparatively lower than with PEC as filler cells some supernatants contained very high levels of GM-CSF as well as the other CSF types (Fig. 2*b*).

CSF has recently been obtained from T-cell hybridomas¹⁶. That normal T cells produce CSF is supported by our experiments with TCGF-dependent cell lines in long-term cultures. Such lines and clones, however, might be aberrant with respect to karyotype, gene expression and regulation of their gene products but this may primarily be the case for T-cell clones growing in medium supplemented with TCGF¹⁷. Therefore, we have complemented our analysis of such clones with T-cell lines and clones growing in long-term culture in the presence of TCGF and antigen. Cells of this type were found to have a normal karyotype (M. Nabholz, personal communication). In addition, our T-cell lines in long-term culture have not shown TCGF-independent growth or tumorigenicity which may indicate a transformed phenotype. As these cells probably represent a highly selected population, results should be compared with those from analysis of T cells in limiting dilution. The following data are therefore in line with our limiting dilution analysis.

Three representatives of T-cell lines in long-term culture are shown in Table 2. PC-AKR clone 29, previously described to produce high amounts of immune interferon on Con A stimulation^{18,19}, has been passaged in permanent culture for over 18 months in the complete absence of antigen and adherent cells and in the presence of TCGF. The alloreactive AKR anti-B6 T-cell lines PK 7.1.1b and PK 7.1.2 grow in the presence of both TCGF and irradiated B6 stimulator cells^{9,20,21}. PC-AKR clone 29 produced GM-CSF and Meg-CSF whereas both alloreactive T-cell lines and clones derived from them (data not shown) produced all four types of CSF after stimulation with Con A (Table 2). These results differed from previous reports where CSF production by continuous T-cell lines was found to be constitutive²² or dependent on the presence of syngeneic accessory cells²³.

We further studied whether antigen-specific recognition is involved in triggering CSF release using the alloreactive AKR anti-B6 T-cell line PK 7.1.2. This line shows antigen-specific proliferation against stimulator cells from mice which carry the *b* allele in the *K* and *I-A* region of the *H-2* complex (Table 3). It can be seen from Table 3 that stimulator cells with the same *H-2* determinants as those that induce proliferation also induce CSF release.

In conclusion, our data indicate that a large proportion of T cells produce CSF on mitogen induction. CSF release is also triggered from T cells during specific antigen recognition. Accessory cells do not seem to be absolutely necessary for CSF production although they might have a potentiating physiological role. Furthermore, the supernatant of a single T-cell clone can contain different types of CSF. However, the production of Meg-CSF, Eo-CSF and E-CSF occurs only concomitantly with GM-CSF. This may indicate that the CSF genes belong to a gene family derived from a common precursor. Indeed, the different CSF types seem to have a striking biochemical similarity²⁴. It is not yet clear whether in physiological conditions of induction of CSF release and of antigen-specific recognition all CSF types are always produced simultaneously or whether separate inductive signals trigger the release of different CSF types to assure a fine tuning of the formation of haematopoietic cells.

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Japanese wild mouse, *Mus musculus molossinus*, has duplicated immunoglobulin $\gamma 2a$ genes

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The immunoglobulin heavy chain loci of the mouse contain eight tightly linked genes that encode the constant region (C) of the immunoglobulin heavy chains (H). As no recombination occurred within the immunoglobulin C_H gene loci among more than 3,000 crosses of mice¹⁻³, the mouse C_H genes were believed to be inherited as a set, designated the *Igh* haplotype. Recent molecular cloning experiments⁴⁻⁹ have demonstrated that the organization of the mouse *Igh* loci of BALB/c (*Igh*^a haplotype) is 5'- C_{μ} -(4.5 kilobases [kb])- C_{δ} -(55 kb)- $C_{\gamma 3}$ -(34 kb)- $C_{\gamma 1}$ -(21 kb)- $C_{\gamma 2b}$ -(15 kb)- $C_{\gamma 2a}$ -(14 kb)- C_e -(12 kb)- C_{α} -3'. The structural analyses of four C_{γ} subclass genes have shown that they are essentially identical in terms of lengths and locations of each of the structural and intervening sequences^{6,10-12}. Comparison of the nucleotide sequences of the $C_{\gamma 1}$, $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes has shown that limited portions of the C_{γ} gene are conserved between compared pairs of the C_{γ} genes¹². The results imply that the nucleotide sequences of the C_{γ} gene segments have been exchanged by recombination among related clustered genes. Similar studies on the $C_{\gamma 2a}$ genes of different haplotypes, *Igh*^a and *Igh*^b, have also suggested that these two types of $C_{\gamma 2a}$ gene have undergone recombinational exchange of a part of the gene sequence¹³. These results imply, in contrast to the previous belief, that frequent recombinational events have taken place within the *Igh* loci and that their nucleotide sequences have been rearranged during evolution. Here we present direct evidence that a Japanese wild mouse, *Mus musculus molossinus*, contains duplicated $C_{\gamma 2a}$ genes while the copy numbers of the neighbouring C_H genes, that is, the $C_{\gamma 1}$, $C_{\gamma 2b}$ and C_e genes, remain constant. The results indicate that the duplicated $C_{\gamma 2a}$ genes of *M. m. molossinus* arose from unequal crossing-over between homologous chromosomes.

During our evolutionary study of immunoglobulin C_H genes (Y.Y. and T.H., in preparation) we found that DNAs of the Japanese wild mouse *M. m. molossinus* had several extra restriction DNA fragments which hybridized with a $C_{\gamma 2b}$ - $C_{\gamma 2a}$ probe, whereas DNAs of many laboratory strain mice had a single fragment for each C_{γ} gene (ref. 6 and Y.Y. and H.T., in

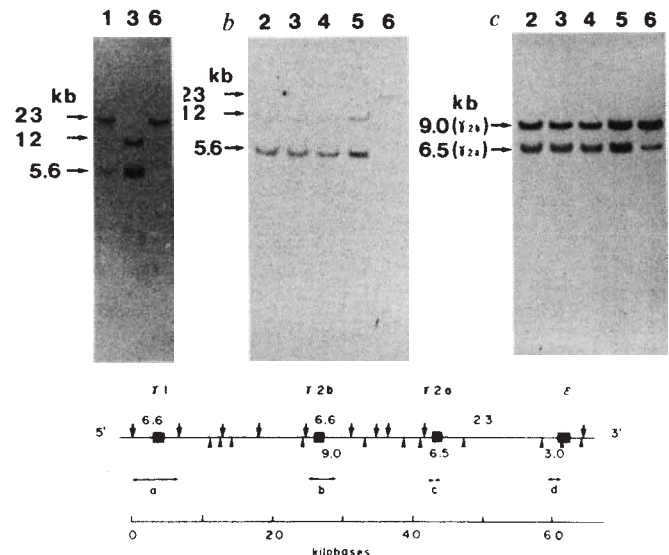


Fig. 1 Southern blot hybridization of *M. m. molossinus* liver DNA with $C_{\gamma 2a}$ or $C_{\gamma 2b}$ - $C_{\gamma 2a}$ gene probe. High molecular weight DNA was extracted from each individual liver of a wild heterozygous *M. m. molossinus* or inbred *M. m. molossinus* mice, or from livers of BALB/c mice as described previously²⁰. We used three individual mice of inbred *M. m. molossinus* which are derived from wild mice trapped at Teine, Hokkaido Island. These mice are designated as Mol. Ten (individual numbers 101, 102 and 103). The other individual mouse of inbred *M. m. molossinus*, designated as MOL. A, is the progeny of wild mice trapped at Anjo, Aichi, Honshu Island. DNAs (1.5 μ g each) were digested with *Eco*RI (a, b) or *Hind*III (c), electrophoresed in a 0.5% agarose gel and transferred to nitrocellulose filters as previously described^{20,21}. Probes used are $C_{\gamma 2a}$ (probe c) for a and b and $C_{\gamma 2b}$ - $C_{\gamma 2a}$ (probe b) for c. Probes were labelled by nick-translation and hybridized as described elsewhere^{20,22}. Origins of DNAs used are as follows: lane 1, a wild *M. m. molossinus*; lane 2, Mol. Ten 103; lane 3, Mol. Ten 102; lane 4, Mol. Ten 101; lane 5, MOL. A; lane 6, BALB/c. A restriction endonuclease cleavage map around the $C_{\gamma 2a}$ gene of the BALB/c mouse and fragments used for probes are shown at the bottom. Data for the restriction map are taken from ref. 4. Structural genes are shown in closed boxes. Numbers above and below the top line indicate the sizes (kilobases) of *Eco*RI and *Hind*III fragments, respectively, which are described in the present study. Horizontal arrows under the top line indicate the fragments used as probes: a, 6.6-kb *Eco*RI fragment of $\gamma 1$ gene¹⁰; b, 4-kb *Xba*I-*Hha*I fragment of $C_{\gamma 2b}$ gene¹¹ which hybridizes to not only the $C_{\gamma 2b}$ gene but also the $C_{\gamma 2a}$ gene^{4,20}; c, 217-base pair *Hae*III fragment of $C_{\gamma 2a}$ gene which contains the 5' 12 base pairs of the hinge region exon and the 3' half of the intervening sequence between the C_H 1 domain and the hinge region exons¹²; d, 2-kb *Bam*HI-*Hind*III fragment of C_e gene⁵. $\downarrow$ and $\blacktriangle$ indicate *Eco*RI and *Hind*III sites, respectively.

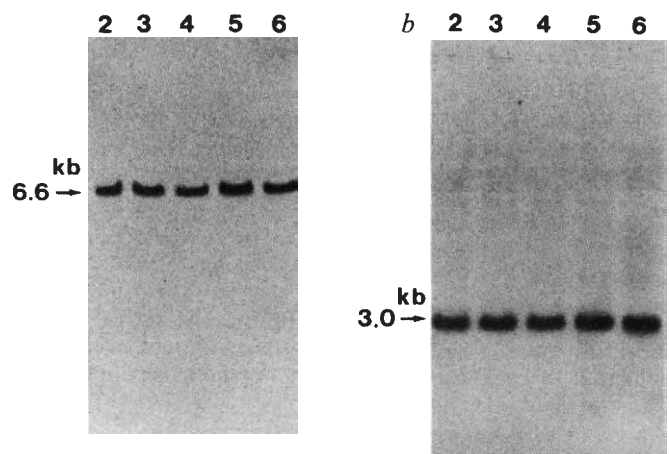


Fig. 2 Southern blot hybridization of *M. m. molossinus* liver DNA with $C_{\gamma 1}$ or C_e probe. DNAs (1.5 μ g each) were digested with *Eco*RI (a) or *Hind*III (b) and electrophoresed, blotted and hybridized as described in Fig. 1 legend except that probes used are $C_{\gamma 1}$ (probe a in Fig. 1) for a and C_e (probe d in Fig. 1) for b. Origins of DNAs used in lanes indicated were the same as described in Fig. 1 legend.



Fig. 3 Restriction map of two cloned $C_{\gamma 2a}$ genes of *M. m. molossinus*. High molecular weight DNA of Mol. Ten 103 was digested with *EcoRI* to completion and the digests were fractionated by electrophoresis in a 0.5% agarose gel. Fragments of 5.6 and 12 kb were collected and ligated to λ gtWES arms as described previously⁹. Ligated DNAs were packaged *in vitro* and screened with BALB/c $C_{\gamma 2b}$ - $C_{\gamma 2a}$ probe (probe b in Fig. 1) as described elsewhere⁴. Two and three identical clones were isolated from 5.6- and 12-kb fragments, respectively. Inserts of λ gtWES-Mol- $\gamma 2a$ -5 (5.6 kb) and λ gtWES-Mol- $\gamma 2a$ -56 (12 kb) are shown by horizontal bars. Closed boxes indicate structure genes. Restriction enzyme cleavage sites are indicated as follows: ↓, *EcoRI*; ▲, *HindIII*; ◆, *XhoI*; †, *BglI*.

preparation). DNA of *M. m. molossinus* had four *EcoRI* fragments (12, 9.3, 5.6 and 1.85 kb) hybridizing with a $C_{\gamma 2b}$ - $C_{\gamma 2a}$ probe in addition to the 23-kb $C_{\gamma 2a}$ and 6.6-kb $C_{\gamma 2b}$ fragments which are shared by BALB/c, C57BL and other inbred strain mice⁶. We used the $C_{\gamma 2b}$ gene fragment (probe b in Fig. 1) as a probe for both $C_{\gamma 2a}$ and $C_{\gamma 2b}$ genes. The $C_{\gamma 2a}$ gene fragments were usually less intense than the $C_{\gamma 2b}$ gene fragment because the $C_{\gamma 2a}$ gene sequence was detected by cross-hybridization to a part of the probe. As the 12- and 5.6-kb fragments were less intense than the 9.3- and 1.85-kb fragments, we presumed that the former fragments are the $C_{\gamma 2a}$ gene.

To determine unequivocally which of the above *EcoRI* fragments are the $C_{\gamma 2a}$ gene, we used as a probe the restriction DNA fragment that contains the 3' half of the intervening sequence between the coding sequences for the C_H1 domain and the hinge region of the $\gamma 2a$ chain (probe c in Fig. 1). This probe has little cross-hybridization with the $C_{\gamma 2b}$ gene¹². As shown in Fig. 1a, DNA of an individual *M. m. molossinus* mouse (which was trapped at Mishima, in Honshu Island) has three *EcoRI* fragments (23, 12 and 5.6 kb) that hybridize with the $C_{\gamma 2a}$ probe. The results seem to indicate that the $C_{\gamma 2a}$ gene is duplicated in this wild mouse, although the presence of heterozygous $C_{\gamma 2a}$ gene cannot be excluded.

We next examined DNAs of four individual mice of *M. m. molossinus* which have been maintained in laboratories by full-sibmating for 11 generations in the Mol. Ten strain and for 23 generations in the MOL. A strain. The Mol. Ten strain was derived from wild mice caught at Teine in Hokkaido Island and the MOL. A strain from those caught at Anjo in Honshu Island. *EcoRI* digestion of DNAs of these *M. m. molossinus* strains gave two DNA fragments (12 and 5.6 kb) which were identical for the two strains, whereas DNA of BALB/c mice had a single *EcoRI* fragment of 23 kb (Fig. 1b). The results strongly support the idea that *M. m. molossinus* has the duplicated $C_{\gamma 2a}$ genes, one of which is located in the 12-kb *EcoRI* fragment and the other in the 5.6-kb fragment. One can still argue the possibility, however, that *M. m. molossinus* has introduced point mutations which have created new *EcoRI* sites,

thus converting the 23-kb fragment into the 12- and 5.6-kb fragments.

To exclude this possibility, we used another restriction enzyme, *HindIII*. *HindIII* digestion of *M. m. molossinus* produced the 9.0-kb $C_{\gamma 2b}$ fragment and the 6.5-kb $C_{\gamma 2a}$ fragment, both of which are identical in size to those of BALB/c DNA (Fig. 1c). However, 6.5-kb bands of the $C_{\gamma 2a}$ gene of *M. m. molossinus* DNAs are about twice as dark as that of the BALB/c DNA. In contrast, the intensity of the $C_{\gamma 2b}$ gene bands (9.0 kb) remains the same among DNAs of *M. m. molossinus* and BALB/c mice.

To examine whether neighbouring C_H genes are duplicated, we analysed the $C_{\gamma 1}$ and C_e genes. As Fig. 2 clearly shows, the intensity and size of the $C_{\gamma 1}$ and C_e genes in these wild mice are indistinguishable from those of BALB/c mice. We have also found that the $C_{\gamma 3}$ gene is not duplicated (data not shown). Previously we showed that the number of the C_{μ} and C_{α} gene fragments of *M. m. molossinus* are identical to those of many laboratory inbred strain mice although the sizes of the fragments vary slightly⁶.

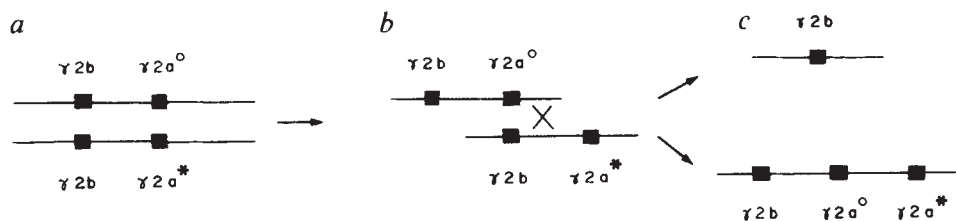
We have further cloned two DNA fragments, each containing a complete $C_{\gamma 2a}$ gene, from DNA of an individual *M. m. molossinus*. One $C_{\gamma 2a}$ gene is located in the 12-kb *EcoRI* fragment and other in the 5.6 kb *EcoRI* fragment (Fig. 3). The restriction sites in the cloned DNA fragments are consistent with those determined by Southern blot analyses.

These results demonstrate that *M. m. molossinus* has a new haplotype which contains the duplicated $C_{\gamma 2a}$ genes. In spite of the fact that these four mice are derived from two independent populations in different islands of Japan, the mice seem to have a similar haplotype, indicating that this haplotype is widely distributed among various local colonies of *M. m. molossinus*.

Lieberman and Potter¹⁴ analysed allotype markers of serum immunoglobulins of homozygous *M. m. molossinus* (Kyushu) and found that this wild mouse had IgG2a^a markers in addition to the IgG1^b, IgG2b^b, IgG2a^b and IgA^b markers. They proposed that this haplotype is created by unequal crossing-over between the *Igh*^a and *Igh*^b haplotypes. Using monoclonal antibodies which can distinguish *a* and *b* allotypes, Herzenberg and his associates (personal communication) also found that two individual mice of inbred *M. m. molossinus* (Mol. Ten strain) have two allotypes of IgG2a, namely IgG2a^a and IgG2a^b. Because the restriction sites around the coding regions of the $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes are well conserved between C57BL (*Igh*^b) and BALB/c (*Igh*^a)^{6,9}, we are unable to correlate the $C_{\gamma 2a}$ gene fragments with serological markers. The restriction map of the cloned $C_{\gamma 2b}$ gene of *M. m. molossinus* is different from those of BALB/c and C57BL (A.S. *et al.*, in preparation). As Fig. 4 shows, it is therefore likely that the duplicated $C_{\gamma 2a}$ genes of *M. m. molossinus* arose from unequal crossing-over between two different haplotypes which must be characterized and identified by nucleotide sequence determination.

Since immunoglobulin allotypes are distributed among various subspecies of *M. musculus*, the allotype seems to have evolved before the divergence of the subspecies of *M. musculus*.

Fig. 4 A possible recombination that created duplicated $C_{\gamma 2a}$ genes with different allotypes. *a*, schematic representation of heterozygous mouse chromosomes containing two different $C_{\gamma 2a}$ genes. $\gamma 2a^*$ and $\gamma 2a^o$ indicate $C_{\gamma 2a}$ genes with different allotypes. *b*, Unequal pairing between homologues at meiosis and unequal crossing-over. As $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes have extensive homology^{8,12}, such a misalignment may occur at meiosis. *c*, Resultant chromosomes. In one chromosome (upper) the $C_{\gamma 2a}$ gene has been deleted, while the other chromosome contains the duplicated $C_{\gamma 2a}$ genes which have different allotypes. The latter chromosome may be an ancestor of the present chromosome in *M. m. molossinus*.



The duplication of the $C_{\gamma 2a}$ gene must have taken place after the divergence of *M. m. molossinus* and *M. m. domesticus*, an estimated one million years ago¹⁵⁻¹⁷. Duplication of immunoglobulin genes has also been suggested in humans by serological studies^{18,19}.

EcoRI digestion of DNA of the first heterozygous *M. m. molossinus* mouse produced three $C_{\gamma 2a}$ gene fragments (23, 12 and 5.6 kb) and three $C_{\gamma 2b}$ gene fragments (9.3, 6.6 and 1.85 kb). It is now clear that the 12- and 5.6-kb $C_{\gamma 2a}$ gene fragments are located on one chromosome and the 23-kb $C_{\gamma 2a}$ gene fragment on the other. We also know that the 6.6-kb $C_{\gamma 2b}$ gene fragment is derived from the chromosome containing the duplicated $C_{\gamma 2a}$ gene. Consequently, the other chromosome should contain two $C_{\gamma 2b}$ gene fragments (9.3 and 1.85 kb) and one $C_{\gamma 2a}$ gene (23 kb). It is not clear whether these two *EcoRI* fragments of the $C_{\gamma 2b}$ gene are due to the gene duplication or

to the introduction of new *EcoRI* sites by point mutation.

The number of C_H genes has been thought to be invariant among inbred strains, although definitive proof is lacking. Previous studies (ref. 6 and Y.Y. and H.T., in preparation) using Southern blot analyses indicate the presence of a single gene for each of the C_{μ} , $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_e and C_{α} genes in many inbred strains including BALB/c, C57BL, AKR, AJ, NZB and SJL. It may be safe to conclude that inbred strains mentioned above have similar sets of C_H genes although they have considerable divergence in the nucleotide sequence.

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Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants

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Neurofibrillary tangles are a prominent feature in the pyramidal cells of the hippocampus and in neurones of the cerebral cortex of people suffering from senile dementia of the Alzheimer type (SDAT)¹. Similar neuronal changes also occur in elderly Down's syndrome patients and to a lesser degree in intellectually normal old people². The tangles are composed of bundles of paired helically twisted 10-13 nm filaments (PHF)^{3,4}, which may be either neurofilaments³ or microtubules⁵. Attempts to isolate tangles from postmortem material have met with some success⁶ but there is disagreement over the molecular weight (M_r) of presumptive PHF proteins enriched in these fractions, with conflicting claims of 50,000 (refs 6, 7) and 20,000 (ref. 8). Initially it seemed that the 50,000- M_r putative PHF protein and tubulin cross-react^{9,10} but this result may have been a serological artefact, due to tangle-associated material in preparations of microtubule proteins used as immunogen and as test antigen¹¹. We have used monoclonal antibodies to show here that neurofilament antigens are present in neurofibrillary tangles.

Monoclonal antibodies were produced as previously described using as immunogen either Triton X-100-insoluble rat brain protein¹² (monoclonal antibodies RT 97, RS 18, 147, 155) or a crude soluble protein fraction (1 h 150,000 g_{av} supernatant) from two pooled hippocampi (SDAT cases U212 and U224, one of which was also studied immunohistochemically; see Table 2) (monoclonal antibody BF 10). Hybridomas secreting antibody to neurofilaments were screened as before¹² and to neurofibrillary tangles by the peroxidase-antiperoxidase (PAP) or by the indirect immunoperoxidase methods on paraffin sections of tangle-rich human brain¹³. Neuropathological samples were selected from postmortem brains of three

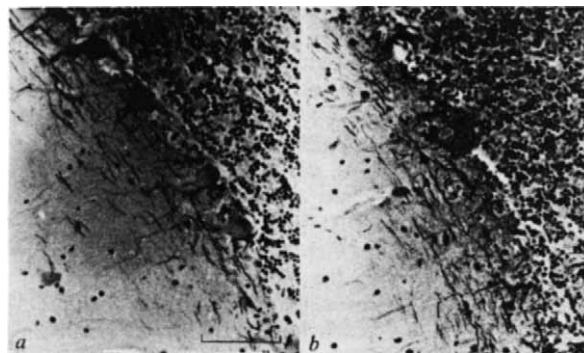


Fig. 1 Human cerebellum (paraffin sections) was stained by the PAP method¹³. *a*, Monoclonal antibody 147; *b*, monoclonal antibody BF 10 (counter-stained with Harris's haematoxylin). Baskets and neuronal fibres reacted strongly. Monoclonal antibodies were used both as tissue culture supernatants (undiluted) and as ascites fluid [diluted 1:1,000 with Tris-buffered saline, pH 7.6 (TBS)]. Normal horse serum (diluted 1:5 with TBS) was used instead of normal goat serum in the preincubation step to eliminate non-specific staining. After overnight incubation at ambient temperature with monoclonal antibodies, sections were washed in TBS (30 min), overlaid with rabbit anti-mouse IgG (H+L; Nordic) diluted 1:200 with TBS (30 min), washed in TBS (30 min), overlaid with goat anti-rabbit IgG (Fc fragment; Nordic) diluted 1:200 with TBS (30 min), washed in TBS (30 min), overlaid by PAP produced in rabbits (Miles) diluted 1:300 with TBS. The reaction was developed with diaminobenzidine (BDH). Scale bar, 100 μ m.

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cases of senile dementia of the Alzheimer type, one elderly Down's syndrome subject and one intellectually normal old subject. We found that five monoclonal antibodies (Table 1) resembled rabbit anti-bovine neurofilament 210,000- M_r polypeptide (anti-210K) sera previously described^{13,14} in their reaction on human cerebellum, as they all reacted with basket cell axons around the Purkinje cells and neuronal fibres in the inner third of the molecular layer (Fig. 1). Monoclonal antibodies BF 10 and 147 and the rabbit anti-210K antiserum were found to react with some neuronal fibres in the cortex and subcortical white matter (Fig. 2a). BF 10 and RT 97 were the only antibodies to react with tangles, and did so in all cases studied (Tables 1, 2); the extent of reaction with tangles resembled that of similar slides after silver or Congo red staining. Initial studies of antibody RT 97 in tissue culture supernatants suggested that the antibody was not tangle reactive, but subsequent studies using ascites fluid showed that, like BF 10, it also stains tangles down to a dilution of ascites fluid of at least 1:2,000. Ascites fluid antibodies RS 18, 147 and 155 do not react with tangles even at 1:200 dilutions whereas all stain neurofilaments at 1:2,000 dilution. The ascites fluid was used at dilutions of at least 1:2,000, as, if more concentrated, background staining of sections becomes appreciable.

PHF can be associated with neuronal perikarya in the form of neurofibrillary tangles or with certain neurites in plaques. As well as staining tangles, BF 10 and RT 97 reacted strongly with numerous distended terminals or neurites which constitute the major part of the senile plaque (Fig. 2c-e). In case T/B, BF 10-positive material was found associated with plaques and only an occasional perikaryon (Fig. 2f); RT 97 was not studied in sufficient detail to know whether it reacted identically with BF 10 in this case. Only a minority of the neurites in plaques reacted with antibodies other than BF 10 and RT 97 (Fig. 2b; Table 1) and none of the antibodies stained amyloid. These results are consistent with our earlier observations of the lack of tangle-reactivity of rabbit anti-bovine neurofilament sera¹³. Absorption of BF 10 with purified bovine neurofilaments (polypeptide pattern shown in Fig. 3a) removed all staining with this antibody.

The monoclonal antibodies were analysed by immunoblotting¹⁵ on purified bovine neurofilaments and on total protein from SDAT and control human hippocampi (Fig. 3). BF 10 and 155 react predominantly with the 155,000- M_r neurofilament protein of bovine neurofilaments and the corresponding polypeptide in human brain. RT 97, 147 and RS 18 bind most strongly to the 210,000- M_r bovine neurofilament protein and the equivalent human polypeptide. Several of the monoclonal antibodies seem to cross-react^{14,16,17} with the 210,000- and 155,000- M_r neurofilament proteins. BF 10 reacts with both the 155,000- and 210,000- M_r polypeptides of bovine neurofilaments (Fig. 3b) but is specific for the 155,000- M_r polypeptide of human neurofilaments (Fig. 3g, n). RT 97 also seems to react with both polypeptides in bovine neurofilaments (Fig. 3d) but in human material may be specific for the 210,000- M_r protein

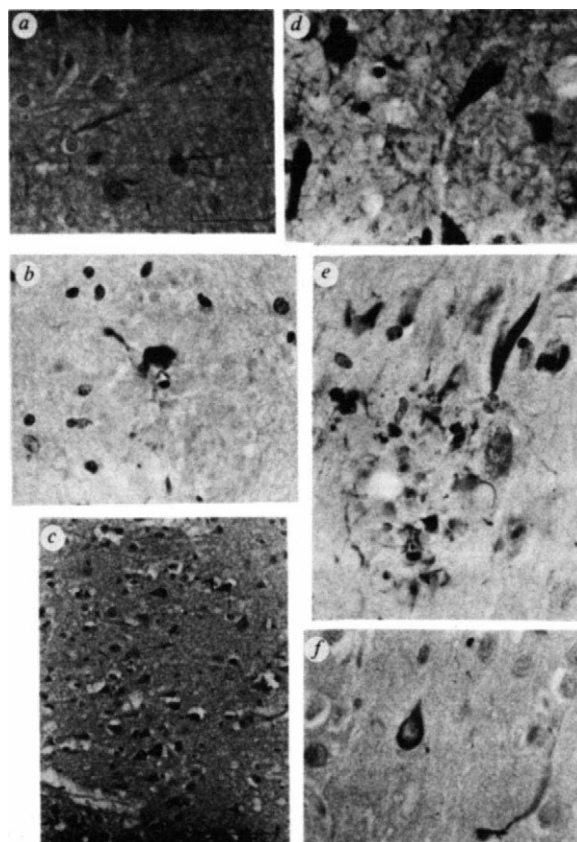


Fig. 2 Reaction of plaques and tangles with monoclonal antibodies 147 and BF 10. *a*, T/A fibres in cortex reacting with 147 (scale bar, 50 μ m; applies to *a*, *b*, *d*-*f*); *b*, U/144 neurites in a plaque reacting with 147; *c*, T/A tangles in pyramidal cells in cortex reacting with BF 10 (scale bar, 100 μ m); *d*, U/303 tangles in pyramidal cells of hippocampus reacting with BF 10; *e*, U/144 tangles and neurites in a plaque reacting with BF 10; *f*, T/B tangles in a pyramidal cell perikaryon reacting with BF 10.

(Fig. 3i, p). RS 18 (Fig. 3e, j, q) reacts similarly to RT 97 but 147 seems specific to the 210,000- M_r polypeptide of both bovine and human neurofilaments (Fig. 3c, h, o). Monoclonal antibody 155 is specific for the 155,000- M_r polypeptide in both species but reacts strongly with several lower molecular weight (50,000-60,000) bands (Fig. 3f, k, r). Antibody BF 10 also reacts strongly with two to four bands just below the 155,000- M_r polypeptide. We have tentatively attributed these extra bands to proteolytic degradation of the major neurofilament proteins; if this is the case, the corresponding antigenic determinants are probably well separated in the 155,000- M_r polypeptide. Other bands, which can be visualized on longer exposure of autoradiographs with all the antibodies (data not

Table 1 Immunohistochemical reaction pattern and antigenic specificities of antibodies

Clone	Immunoglobulin subclass	Reaction with neurofibrillary tangles	Reaction with human senile plaques	Reaction with human neurofilaments*	Neurofilament polypeptide with major antigenic determinant (M_r)
BF 10	IgG2a	+	N	+	155,000
RT 97	IgG1	+	N	+	210,000
RS 18	IgG1	-	n	+	210,000
147	IgG1	-	n	+	210,000
155	IgG1	-	n	+	155,000
BF 10-absorbed	-	-	-	-	ND
Rabbit anti-210K neurofilament sera	-	-	n	+	210,000, 155,000

N, most neurites; n, occasional neurites. ND, not determined.

*Determined by characteristic staining pattern on cerebellum.

Table 2 Comparison of immunohistochemical reaction pattern of two monoclonal antibodies on five different postmortem specimens

Case	Area examined	BF 10	Reaction pattern 147, rabbit anti-210K antiserum
U/224 (SDAT)	DG, H	T, N	n
U/303 (SDAT)	DG, H	T, N	n
T/A (SDAT)	NC, SWM	T, N, A	n, A
U/144 (Down's syndrome)	DG, H	T, N	n
T/B (old age)	NC, SWM	T, N, A	n, A

DG, dentate gyrus; H, hippocampus; NC, neocortex; SWM, subcortical white matter. T, neuronal perikaryal tangles; A, axons; N, most neurites in senile plaques; n, occasional neurites in senile plaques.

shown), are being investigated. Immunoblotting did not reveal any outstanding differences between normal and SDAT neurofilament antigens with any of the antibodies used, including BF 10 and RT 97. Differences such as the relative sharpness and intensities of individual bands are probably the result of postmortem degradation and variability in the efficiency of blotting and would only be significant if confirmed in analyses of many different postmortem specimens. The neurofilament proteins detected by the antibodies on the immunoblots are minor constituents of the total human hippocampal proteins present as neurofilament bands in the stained tracks (Fig. 3*l, m*) are not obviously visible; this attests to the sensitivity and specificity of the antibodies for immunochemical analysis of neurofilament antigens (neurofilaments are only a minor component of the total protein composition of grey matter even of fresh rat brain although white matter is relatively rich in neurofilaments).

Neurofibrillary tangles must share at least two antigenic determinants with neurofilaments as the immunoblots analysis has shown RT 97 and BF 10 to be clearly directed at different sites of the neurofilament structure and possibly different neurofilament polypeptides (Table 1). Some tangles contain a mixture of apparently normal neurofilaments and PHF^{18,19} and are occasionally composed of bundles of straight filaments (B.E.T., unpublished) but the strength of the reaction with the BF 10 and RT 97 monoclonal antibodies with most of the tangles suggests that the reactive component is not simply due

to inclusions of neurofilaments. Ultrastructural studies with this antibody are required to confirm this. As other monoclonal antibodies and rabbit antisera which react with normal human neurofilaments fail to stain tangles, any neurofilaments present in the PHF (or straight filaments) must be structurally different. The differential reactivity of RT 97 in tissue culture supernatants with tangles and normal neuronal fibres suggests that the corresponding antigenic determinant may be partially affected in tangles resulting in a failure of RT 97 to bind when the antibody concentration is low. The pattern of reactivity with the panel of antibodies is consistent for all tangles of a given case and for all five cases studied (three SDAT, one Down's syndrome, one normal old age), which implies that any change in neurofilament integrity is a precisely defined event and not a random breakdown of structure. Immunoblotting so far has failed to reveal any gross differences in molecular weight of neurofilament antigens from control and SDAT tissues and suggests that an altered antigenicity may be the result of either an intrinsic conformational change in the filament protein or a different packing and/or masking of antigenic determinants. BF 10 and RT 97 monoclonal antibodies should be useful new reagents for probing the chemical composition of neurofibrillary tangles.

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Note added in proof: Selkoe *et al.*²⁰ have claimed that PHF are insoluble in SDS which would therefore make it difficult to analyse these proteins by SDS-gel electrophoresis and immunoblotting. This same group²¹ have also recently published evidence that the 20,000-M_r protein previously found to be enriched in the PHF fraction⁸ is, in fact, myelin basic protein.

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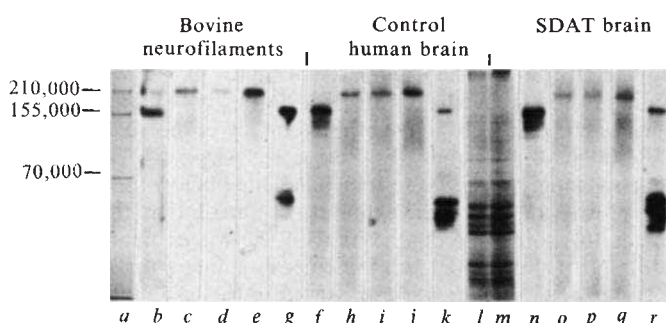


Fig. 3 Immunoblots showing the antigenic specificities of all five monoclonal antibodies on isolated bovine neurofilaments (a-f); total hippocampal proteins of control human brain (g-l); total hippocampal proteins of SDAT brain (m-r). a, l, m Are stained with Coomassie brilliant blue and are tracks cut from SDS slab gels²². The remainder of the tracks, with identical sample loadings, were blotted and processed essentially as according to Towbin *et al.*¹⁵ on to nitrocellulose sheets which were then cut into strips and each strip incubated with a different antibody followed by ¹²⁵I-labelled rabbit anti-mouse immunoglobulins. The autoradiographs are aligned exactly with the stained gel strips to show the position of the labelled antigens present in the total sample proteins. BF 10 (b, f, n); 147 (c, h, o); RT 97 (d, i, p); RS 18 (e, j, q); 155 (g, k, r). Ascites fluid for the incubations with the nitrocellulose blots was diluted as follows: BF 10—1:10² (b), 1:5×10⁴ (f, n); 147—1:4×10³ (c), 1:10⁴ (h, o); RT 97—1:1.5×10³ (d), 1:2×10⁴ (i, p); RS 18—1:2×10³ (e), 1:5×10³ (j, q); 155—1:2×10³ (g), 1:5×10³ (k, r). The molecular weight of the individual bovine neurofilament triplet polypeptides are indicated at the left-hand side.

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A model for the molecular requirements of immunoglobulin heavy chain class switching

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Heavy chain immunoglobulin gene expression requires two independent DNA recombination systems¹⁻³. The first DNA rearrangement event (V-D-J recombination) involves the formation of a complete variable region (V_H) gene in pre-B lymphocytes³⁻⁶. The second series of recombination events allows for a switch in heavy chain constant region class from μ to either $\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2a$, ϵ or α ¹⁻³. These recombination events occur in intervening sequences but do not alter or generate new coding information which is a common feature of V-D-J unions¹⁻³. Tandemly repeated DNA sequences, spanning ~2-5 kilobases (b), are localized ~1.5 kilobase pairs 5' of each C_H gene and have been termed C_H switch (S) regions^{2,7}. Two short, simple sequences that are shared by all switch region repeats (GAGCT and GGGGT) have been proposed to facilitate C_H switching by homologous DNA recombination⁸. Unlike other switch regions, a large portion of the S _{μ} region (3 kb) consists of essentially pure tandem repeats of GAGCT which are occasionally interrupted by a GGGGT⁸. However, the precise sites of switch-recombination in S _{μ} are generally located considerably 5' of this genetically unstable stretch^{9,10} of GAGCT repeats (Fig. 1). We now report the properties of a novel C_H S region sequence, YAGGTTG, which is 5' of switch recombination sites and preferentially repeated in S regions.

The nucleotide sequences in the immediate vicinity of the switch-recombination sites used by 10 rearranged C_H genes are depicted in Fig. 2. Germ-line S _{μ} sequences are all referred to as 'donor sites' because all functional switch events must originate 5' of the C _{μ} gene and then for simplicity all other germ-line S_H sites are termed 'acceptors'. Acceptors may also serve as

donors when multiple recombination events result in a functional switch. Abortively rearranged C_H genes can either be generated by inter-switch region acceptor recombinations (that is, as with S _{$\gamma 3$} and S _{$\gamma 2b$} in MPC11)¹¹ or by the recombination of non-immunoglobulin-associated DNA sequences (that is, non-switch region DNA) with an S_H region^{12,37,38}. The only common feature at or near all the recombination sites shown in Fig. 2 is the presence of a prevalent heptamer sequence, YAGGTTG. Several expressed C_H genes use an analogue of YAGGTTG in the identical sequence environment, suggesting that these may constitute preferred switch sites. Analogues of this prevalent heptamer are occasionally located 3' of some of these recombination sites. However, GAGCT and GGGGT sequences are absent in 9 of these 21 DNA sequences. The poorest YAGGTTG analogues (4/7 matches) are utilized by the S _{$\gamma 2b$} acceptor in MOPC 141 and the S _{μ} donor of MC101 (a $\gamma 1$ producer). However, the four matched nucleotides are contiguous in both cases. Therefore, we assume that the minimum criterion for a YAGGTTG sequence analogue is a 4/7 match with four contiguous matched nucleotides. As most of these putative recognition sequences are not perfect matches to a YAGGTTG heptamer, we analysed other properties of YAGGTTG-like sequences which might support the idea of their specific involvement in the C_H switching mechanism.

The majority of switch events appear to occur 8-16 nucleotides 3' of this common sequence (Fig. 3). However, four of the rearrangement events shown in Fig. 2 occur within this prevalent sequence. Two of these examples are acceptor sites of abortively rearranged genes ($\gamma 2b$ in MPC11, and α in MOPC 603)^{11,12}. One of these events occurred within intervening sequence (IVS) I of the C _{$\gamma 1$} gene which resulted in a C_H1 domain deletion¹³. The fourth and last case ($\gamma 2b$ in MOPC 141) occurred 1.0 kb beyond the 3' boundary of the repetitive S _{$\gamma 2b$} acceptor region^{3,14}. The expressed $\gamma 2b$ gene in MOPC 141 is the only known example of a switch that occurs beyond the 3' boundary of a repetitive switch region. We have failed to observe a reoccurrence of this rare event in any of 13 different $\gamma 2b$ -producing plasmacytomas (R.B.L. and K.B.M., in preparation). The significance of the preference for 8-16 nucleotides between a YAGGTTG analogue and the actual switch site will require further confirmation from analyses of other functionally switched C_H genes.

YAGGTTG analogues are repeated in switch regions. Twenty-eight YAGGTTG-like sequences are located in the

Table 1 YAGGTTG (Y = CorT) matches in five switch regions of heavy chain constant region genes

Switch region	Length (kb)	YAGGTTG matches				Total	Prevalent matches to YAGGTTG						
		7/7	6/7	5/7	4/7		Y	A	G	G	T	T	G
S _{μ}	5.20	1	8	8	15	32	71	53	82	100	82	88	82
S _{$\gamma 3$} 5'	0.56	0	5	3	8	16	Y	A	G	G	Y	T	G
							100	88	88	100	75	63	63
S _{$\gamma 3$} 3'	0.85	3	9	21	7	40	Y	A	G	G	Y	T	G
							88	88	100	100	70	58	67
S _{$\gamma 1$}	0.59	1	4	5	12	22	C	A	G	G	Y	T	G
							90	100	100	100	80	80	50
S _{$\gamma 2b$}	1.60	0	14	5	21	40	Y	A	G	G	T	G	G
							90	100	95	100	74	79	95
S _{α}	1.40	6	30	16	2	54	T	A	G	G	Y	T	G
							79	81	98	100	88	94	81
Totals	10.2 S _D = 5.2 S _A = 5.0	11	70	58	65	204	Y	A	G	G	Y	T	G
							86	83	96	100	80	71	76
C _H coding regions* (without IVS I)	6.8	0	3	40	72	115	Y	A	G	G	T	N	N
							88	78	95	100	70		

The minimum criteria for a match is 4/7 with all four nucleotides contiguous and all other matches (that is, 7/7, 6/7, 5/7) must also have four contiguous nucleotides. The switch region lengths only refer to sequenced DNA. S _{μ} ^{3,8}, S _{$\gamma 3$} 5'¹¹, S _{$\gamma 3$} 3'²⁷, S _{$\gamma 1$} ¹¹, S _{$\gamma 2b$} ¹¹ and S _{α} ⁷. Prevalent matches to YAGGTTG were determined by only tabulating the 7/7, 6/7 and 5/7 matches. 4/7 matches only seem to be rarely used in functional C_H genes (Fig. 2) and the high probability to detect randomly such a non-specific match (a 1/51 chance) tends to randomize unnecessarily the resultant consensus sequences. The fit of a particular nucleotide is indicated as the percentage of its incidence in all 7/7, 6/7 and 5/7 matches combined. S_D refers to the entire S _{μ} region and S_A refers to all other S regions.

* Data obtained from C _{μ} ²⁸, C _{$\gamma 1$} ²⁹, C _{$\gamma 2b$} ^{30,31}, C _{$\gamma 2a$} ³² and C _{α} ³³ with IVS I contribution removed. N, any of the four nucleotides.

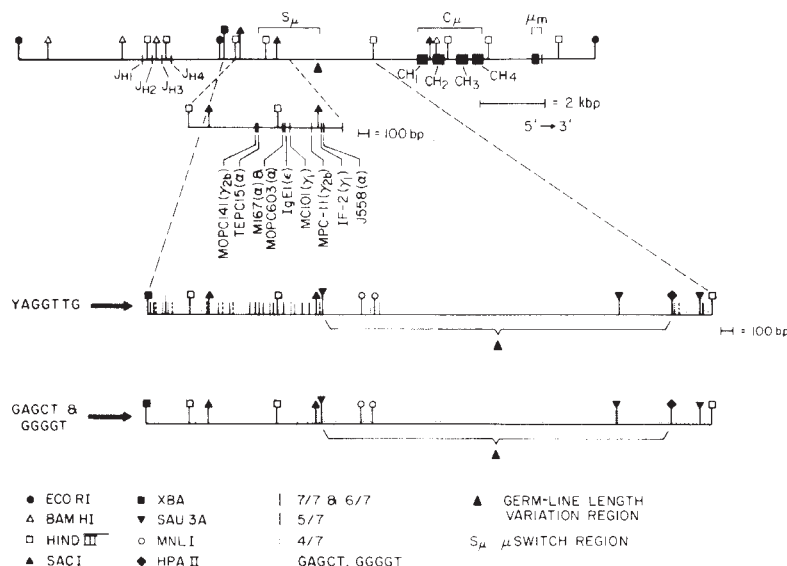


Fig. 1 The relative locations of C_H gene switch sites and two classes of hypothesized recognition sequences are indicated 5' of the mouse C_μ gene. Y represents a C or a T. The nucleotide sequence of the S_μ region were extracted from other publications^{3,8}. The location of the unstable S_μ length variation region that consists of a relatively pure stretch of GAGCT and GGGGT sequences was determined from other published work^{9,11} as well as our unpublished data. MOPC 141 (refs 3, 14), TEPC 15 (ref. 7), M167 (ref. 34), MOPC 603 (ref. 7), Ig E1 (ref. 8), MC101 (ref. 35), MPC 11 (ref. 36), IF-2 (ref. 14) and J558 (L.H. and K.B.M., in preparation).

immediate vicinity of all known S_μ sites used in C_H gene switches and S_H acceptor regions contain numerous repeats of YAGGTTG-like sequences (Table 1). Consensus sequences prepared for YAGGTTG analogues found in different switch regions closely resemble the YAGGTTG prototype sequence. Analogues of this recognition sequence are at least as prevalent as the common GAGCT sequences⁸. The S_μ region is particularly rich in YAGGTTG-like sequences possessing 36 out of 54 examples that are either 7/7 or 6/7 matches in only 1,400 base pairs⁷.

YAGGTTG sequences are not repeated in C_H coding regions. If similar amounts of C_H coding DNA (8.3 kb) and C_H switch region DNA (10.2 kb) are compared, the C_H coding regions are found to contain less than 10% of the total number of 7/7

and 6/7 YAGGTTG matches found within all switch regions (Tables 1, 2). The distribution of YAGGTTG analogues in C_H coding regions is below random expectations (Table 2). Indeed, these sequences are present in random amounts in three mouse globin genes^{15,16} and in the entire J_K - C_K locus^{17,18}. A lower frequency of YAGGTTG-like sequences in C_H coding regions may be selected for to avoid aberrant recombination events. More than 50% of the best YAGGTTG matches, that are found in C_H coding regions, occur in IVS I. If these are excluded from the C_H coding region analysis, the 3' end of the YAGGTTG-like heptamer appears random and gives a poor YAGGYNN composite (Table 1). These observations may be relevant to the phenomenon of rare C_H domain deletions in defective or mutant chains in mice¹⁹ and humans²⁰. Unlike the results observed for the entire C_H coding region, the presence of YAGGTTG analogues in IVS I agrees well with random expectations (Table 2).

We have compiled here considerable evidence for the involvement of a YAGGTTG-like sequence in the mechanism of C_H switch-recombination: (1) YAGGTTG-like sequences are predominant features of all functional switch sites so far identified. (2) YAGGTTG is 5' of the switch sites of both donor and acceptor sites for all C_H switches in a total of 21 examples. (3) Switch-recombination sites preferentially occur 8–16 nucleotides 3' of a YAGGTTG analogue. (4) YAGGTTG-like sequences are preferentially repeated in all S_H acceptor regions but appear unique to the S_μ donor region. (5) YAGGTTG analogues are present below random expectations in C_H domains and their IVSs except IVS Is.

As a model for C_H switch-recombination, we suggest that a YAGGTTG sequence or its close analogue acts with tandem repeats of a GAGCT sequence to mediate an efficient C_H gene switch. The S_μ donor region appears different and not only seems to require a YAGGTTG-like sequence close to the 5' side of the switch site but also to possess a very large region of GAGCT tandem repeats located considerably 3' of the actual switch site. The presence of 3 kb of GAGCT repeats in S_μ ⁸, the conservation of this repetitious region in mice and humans^{21–23} and the existence of GAGCT and GGGGT in switch region consensus sequences⁸ collectively argue that YAGGTTG analogues, as well as GAGCT repeats, are required for an efficient C_H switching process. The need for repeats of both YAGGTTG analogues as well as GAGCT and GGGGT sequences in switch acceptor regions may reflect the pairing process that allows for the proper alignment of C_H genes for an accurate switch event. After the initiation of this pairing event, a switch could perhaps occur close to any YAGGTTG analogue. However, this idea would not rule out that low-frequency switching events may not require the GAGCT

Table 2 Observed frequencies of YAGGTTG sequences compared with their expected random frequencies

	No. YAGGTTG matches			
	7/7	6/7	5/7	4/7
C_H coding regions (8.3 kb)	0 (1)	6 (14)	58 (81)	105 (163)
C_H IVS I only (~1.5 kb)	0 (0)	3 (3)	18 (15)	33 (30)
C_H coding regions without IVS I (6.8 kb)	0 (0.8)	3 (12)	40 (67)	72 (133)
BALB/c α - and β -type globin genes (~5.0 kb)	3 (0.6)	18 (9)	51 (49)	87 (98)
Within 50 bp 5' of J_K and J_H (0.450 kb)	0 (0)	3 (0.8)	8 (4)	15 (9)
S_μ (without GAGCT repeat region; 2.2 kb)	1 (0)	9 (4)	17 (22)	30 (40)
All S regions (without S_μ GAGCT repeat region; 7.2 kb)	11 (1)	81 (12)	139 (70)	202 (140)

The total number of each of the four different categories for YAGGTTG matches are compared with their number derived from random expectations. The random probabilities used for 4/7, 5/7, 6/7 and 7/7 matches to YAGGTTG are 1/51, 1/102, 1/585 and 1/8192 respectively. Statistical probabilities were calculated with the assumption that the four matched nucleotides in the YAGGTTG sequence must be contiguous. Numbers in parentheses indicate the random expectation for the YAGGTTG analogue in a similar size sequence. YAGGTTG analogues were also observed to be random in the J_K - C_K and J_H loci and in the μ - δ intragenic region. C_H coding regions^{28–33}, BALB/c α - and β -type globin genes^{15,16}, J_K locus^{17,18}, J_K - C_K intragenic region and C_K ²⁷, J_H locus²⁷, S_μ ^{3,8}, other S regions^{7,11,27}.

repeats. This would be the case for the $\gamma 2b$ gene in MOPC 141 and the C_H1 domain deleted $\gamma 1$ gene in IF-2 in which the switch acceptor sites have no GAGCT repeats. When considering recombination events associated with immunoglobulin genes, one must also be aware that rare V-J recombinations can occur with the participation of a conserved heptamer sequence in the absence of a nanomer sequence^{18,24}.

We consider this a working hypotheses which may stimulate further studies on C_H switch sites. The examination of other switch recombination events may confirm the role of the YAGGTTG-like sequences. One testable prediction of our model would be that C_H switches occurring in tissue culture variants of myeloma cell lines²⁵ utilize YAGGTTG analogues. A $\gamma 2b \rightarrow \gamma 2a$ switch in an MPC 11 switch variant has recently been shown to result from a DNA deletion event which encompasses the switch site of the $\gamma 2b$ -producing, parental MPC 11 cell and the entire $\gamma 2b$ -coding region²⁶. We suggest that the endpoints of this deletion event may occur close to the 3' side of YAGGTTG analogues. This type of secondary or variant cell switch would be an infrequent or rare event since the S_{μ} GAGCT repeat region is completely deleted on forming the original functional MPC 11 $\gamma 2b$ gene and is therefore unavailable for the secondary switch event. A direct functional test of the molecular requirements for C_H switching could use a gene transfer approach that would use B lymphocytes or switch variant myeloma cells as recipients of genetically modified,

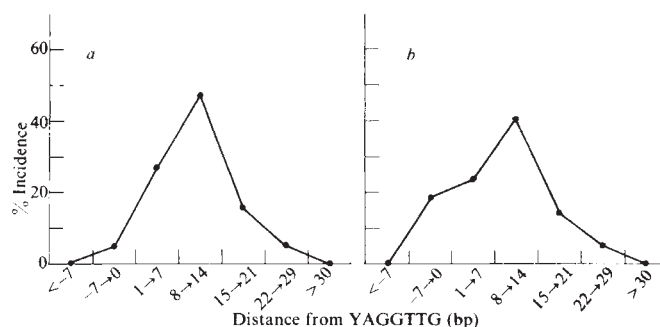


Fig. 3 C_H gene switch recombination sites are not randomly distributed around the YAGGTTG sequence. The percentage of examples of C_H switch events that occur at 7 bp intervals from the prevalent YAGGTTG recognition sequence are depicted as a function of this distance. The recognition sequence is in the $-7 \rightarrow 0$ region of the graphs. *a*, Normally rearranged C_H genes. The abnormal C_H genes IF-2 $\gamma 1$, MPC11 UE $\gamma 2b$ and MOPC 603 UE α are not included in the analysis. *b*, All rearranged C_H genes. All 21 examples presented in Fig. 2 are used for the analysis.

multi- C_H gene substrates whose germ-line switch regions have been deleted or specifically mutagenized.

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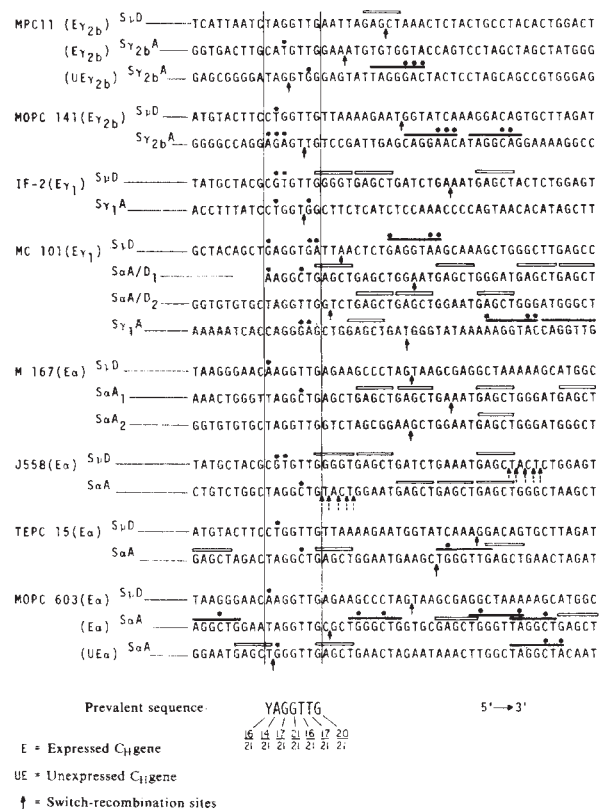


Fig. 2 Nucleotide sequences of the germ-line donor and acceptor environments of eight C_H gene switches and two aberrantly rearranged C_H genes. YAGGTTG sequences that are found in all switch-recombination events, and therefore hypothesized to be important recognition sites, lie between the two vertical lines. Arrows indicate the precise points of DNA breakage. Overhead solid bars indicate the locations of YAGGTTG analogues outside the hypothesized YAGGTTG recognition site. Overhead open bars indicate the locations of GAGCT and GGGGT sequences. Nucleotides in YAGGTTG analogues which do not match their counterparts in the prevalent sequence are marked with overhead dots. MPC11 E $\gamma 2b$ (ref. 16), MPC11 UE $\gamma 2b$ (ref. 11), MOPC 141 E $\gamma 2b$ (refs 3, 14), IF-2 E $\gamma 1$ (ref. 15), MC101 E $\gamma 1$ (ref. 35), M167 E α (ref. 34), J558 E α (L.H. and K.B.M., in preparation), TEPC 15 E α ⁷, MOPC 603 E α ⁷ and MOPC 603 UE α ¹³.

Complete amino acid sequence and maturation of the mouse submaxillary gland renin precursor

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Renin, a protease which cleaves the prohormone angiotensinogen, releasing angiotensin I, has a major role in regulating blood pressure and electrolyte balance. However, the primary structure and the active structure of the enzyme remain unknown. The presence of an inactive form of the enzyme, activable by partial proteolysis, has led to the proposal that renin is synthesized as an inactive precursor¹. Kidney juxtaglomerular cells constitute the principal source of renin, but its concentration in this tissue is too low to allow determination of its primary structure by conventional techniques. In some strains of mice, the male submaxillary glands synthesize and secrete very large amounts of an enzyme which appears indistinguishable from renal renin by immunological and physicochemical criteria^{2,3}, thereby representing a unique opportunity to delineate the detailed features of renin. We now present the analysis of bacterial DNA recombinant clones harbouring an essentially complete transcript of renin mRNA isolated from the mouse submaxillary gland. The deduced amino acid sequence shows that renin is synthesized as a 44,209 molecular weight (M_r) precursor. Comparison with other known acid proteases shows a good alignment of renin and acid protease sequences in the neighbourhood of residues involved in the active site. N-terminal sequence analysis of renin reveals that the precursor is converted by a protein cleavage into active renin consisting of two chains of M_r 35,000 and 3,000.

We have previously described the construction of cDNA clone pRn5.3 which contains ~1,100 nucleotides of a mouse renin mRNA sequence⁴. The presence of a poly(A) extension at one end of pRn5.3, as revealed by nucleotide sequence analysis, indicated that the cDNA sequence in this clone corresponds to the 3' portion of renin mRNA. A clone, designated pRn4.7, containing a sequence 5' upstream of pRn5.3 cDNA, has been isolated (see Fig. 1 legend), and contains ~1,250 nucleotides of renin mRNA sequence. As clones pRn5.3 and pRn4.7 share an identical segment of ~900 nucleotides in their central portion and as pRn5.3 codes for the 3'-terminal region of renin mRNA, together they harbour an essentially complete transcript of renin mRNA. The nucleotide sequence of cDNA inserts was determined using the partial chemical degradation method of Maxam and Gilbert⁵ in conditions previously described⁶. The strategy for sequencing pRn5.3 and pRn4.7 plasmids is presented in Fig. 1a, while Fig. 1b shows the nucleotide sequence of cDNA in pRn5.3 and pRn4.7; the sequence is 1,424 nucleotides long.

The sequence bears one open reading frame of 1,203 nucleotides, the two other reading frames containing, respectively, 17 and 20 nonsense codons. A search for the ATG translation initiating codon in the open reading frame near the beginning of the 5' region revealed two ATG codons located 41 and 56 nucleotides from the 5'-terminal region of pRn4.7 cDNA. If the first ATG codon is the starting codon, the amino-terminal sequence of renin precursor would contain 3 basic residues followed by 10 hydrophobic amino acids. Such a sequence is

very similar to the signal sequences observed at the amino-terminal end of most precursors of secreted proteins⁷, making it likely that the first ATG codon is the starting codon.

As the endoprotease responsible for the cleavage of signal segments splits the peptide bond on the carboxy-terminus side of small uncharged amino acids and as the residues located in the neighbourhood of the cleavage site exhibit a high potential for making β -turns or aperiodic conformations⁸, we propose that the cleavage site for preprorenin probably lies after Cys 18. However, two other cleavage sites—after Ser 21 and Gly 25—cannot be ruled out.

The coding sequence of renin mRNA is 1,203 nucleotides long and ends with the translation stop codon UAA. The deduced amino acid sequence gives a molecular weight for the renin precursor of 44,209. The 3'-untranslated region, which is 180 nucleotides long, contains four more potential stop codons in the same reading frame. The consensus sequence proposed by Proudfoot *et al.*⁹ AATAAA, occurred 12 nucleotides before the polyadenylation site.

Enzymatic studies have shown that renin shares certain catalytic features with acid proteases^{10,11}. James *et al.*¹² have proposed a mechanism for the catalytic hydrolysis of peptide bonds by acid proteases involving the proton shared by Asp 32 and Asp 215 of penicillopepsin as the electrophilic component and Tyr 75 as a charge relay element. These authors have shown that, on substrate binding, Tyr 75, originally bound to Trp 39, is involved in a conformational change.

We aligned the primary amino acid sequences of some pepsin family proteins—bovine and porcine pepsinogens^{13,14}, bovine prochymosin¹⁵ and penicillopepsin from *Penicillium janthinellum*¹⁶—with the renin sequence so as to maximize the homology between proteins. As calculated from Fig. 2, a homology of 37–24% exists between these acid proteases and renin. In particular, the cysteine residues occur within the renin chain at almost identical positions to those in the pepsinogen chain, suggesting the same arrangement of the disulphide bridges, and similarities in tertiary structure for renin and acid protease molecules.

Figure 2 shows that the two regions of highest homology between renin and acid proteases are those surrounding the two renin aspartyl residues Asp 101 and Asp 286, corresponding to Asp 32 and Asp 215 of penicillopepsin. Furthermore, the two residues Tyr 75 and Trp 39 are conserved, occurring, respectively, at positions 146 and 108 of the renin chain. As mechanistic features of proteases indicate that the ways in which residues of a protein attack peptide bonds are limited, this homology in the active site of renin suggests a detailed analogy in catalytic mechanism. Our results thus indicate that renin and acid proteases have arisen by a process of divergent evolution from a single ancestral prototype¹⁷.

To establish the relationship between the renin precursor and the mature active enzyme, we have determined the N-terminal sequence of pure active renin¹⁸. Although renin appeared homogeneous on analytical HPLC and SDS-gel electrophoresis, a double sequence was obtained. After reduction by mercaptoethanol, the molecular weight shifted from 38,000 to 35,000 and a faint band appeared at the front of the gel. A similar observation was reported by Suzuki *et al.*¹⁹. The double sequence and the decrease in molecular weight following reduction suggested that renin exists as two chains linked by a disulphide bridge. Therefore, renin was reduced, carboxymethylated and the two chains separated by gel filtration and subjected to automated Edman degradation; their N-terminal sequences are shown in Fig. 3.

The N-terminal sequence of peptide A, corresponding to the main protein band, determined for 21 positions, coincides with the portion of renin starting at position 64, and the sequence of peptide B, corresponding to the minor band, determined for 21 positions, coincides with the portion of the enzyme starting at position 354. We propose the model of renin processing shown in Fig. 4. The peptide signal is first removed from the renin precursor by membrane processing enzymes, leading to

Fig. 1 *a*, Bacterial cloning and sequencing strategy of recombinant plasmids. Comparison of renin mRNA size, ~1,600 nucleotides as determined previously⁴, and 1,100-nucleotide cDNA insert in pRn5.3 shows that ~400 nucleotides are required to complete the sequence of the renin message (if we assume that the poly(A) track is 100 nucleotides long). To clone the 5' portion of renin mRNA, partially purified renin mRNA was used as a template for reverse transcription²³. The cDNA obtained was converted into double-stranded S₁ nuclease-resistant DNA²³. Double-stranded cDNA of 1.2–1.7 kilobases was eluted from a 3.5% acrylamide gel and inserted into the *Pst*I site of pBR322 by the oligo(d(CG)) tailing method²³. Host bacteria were transformed and colonies were screened by hybridization with the nick-translated cDNA insert of pRn5.3 as a probe. The recombinant plasmid containing the largest 5' portion of renin message was chosen for further analysis and designated pRn4.7. For sequencing experiments, sites of endonuclease cleavage are indicated at the top. As shown by the *Pvu*I site position of the pBR322 plasmid, the recombinant plasmids pRn5.3 and pRn4.7 are inserted in inverse orientation at the *Pst*I site of vector DNA. DNA fragments were labelled either at the 5' end (—) or at the 3' end (---). Arrows indicate the direction and extent of nucleotide reading. *b*, Nucleotide sequence of mouse submaxillary renin cDNA as deduced from the sequence of plasmids pRn5.3 and pRn4.7. The amino acid sequence is that predicted from the nucleotide sequence.

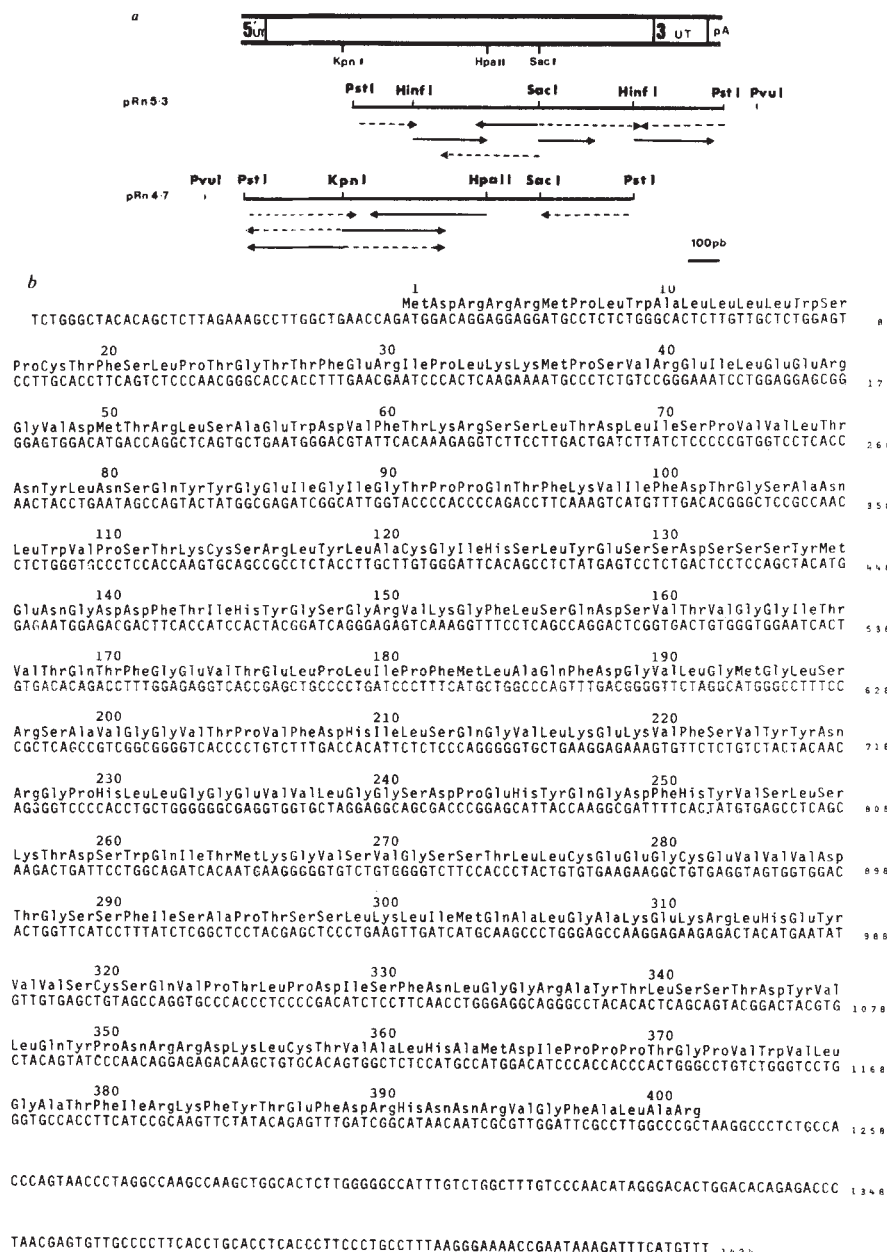


Fig. 2 Comparison of mouse submaxillary renin and acid proteases primary sequences. The upper line gives the predicted amino acid sequence of renin, the four lower lines give the amino acid sequences of bovine and porcine pepsinogens, bovine prochymosin and penicillopepsin from *P. janthinellum*. Data are from Dayhoff²⁴. Only the amino acids which differ from the renin chain are shown. Homologous positions are indicated by dashes. Deletions are indicated by parentheses. The single code for amino acids is given in ref. 24.

A chain S S L T D L I S P V V L T N Y L N S Q Y Y
 B chain D K L C T V A L H A M D I P P T G P V W

Fig. 3 Amino-terminal sequence analysis of renin A and B chains; 30 nmol each of chain A and chain B were analysed by automated Edman degradation in a Beckman sequencer 890C exactly as described elsewhere²⁵.

the formation of prorenin. The prorenin is converted into active mature renin by two proteic cleavages, each occurring after two basic residues: Lys 62–Arg 63 and Arg 352–Arg 353. The exact length of the A and B chains is not known because the carboxyl terminus of the two chains has not been determined. However, the molecular weight of the A chain as determined by SDS-gel electrophoresis (35,000) is close to that calculated from the deduced amino acid sequence (31,347). The addition to A and B chain (5,379) M_r gives a M_r of 36,698, close to that found by SDS-gel electrophoresis and equilibrium sedimentation, making extensive processing of the C-terminus unlikely.

Our model is based on the presence of a disulphide bond between the A and B chains. Cys 4 present in the B chain (Cys 357) is probably linked to Cys 320 of the A chain, by analogy

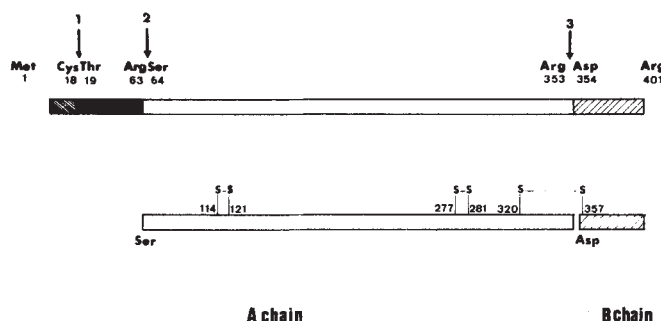


Fig. 4 A schematic representation of the possible maturation process of submaxillary gland renin. The model involves three cleavages: (1) removal of the peptide signal by membrane processing enzymes; (2), (3), conversion of the prorenin obtained into active mature renin by two cleavages occurring after a dibasic peptide and giving rise to the A and B renin chains linked by a disulphide bond.

with pepsin. Similarly, because the position of the cysteines is conserved in the acid protease family, it is likely that there is a disulphide bond between Cys 114 and Cys 121, and between Cys 277 and Cys 281.

Comparison of this model with the primary structures of carboxyl proteases and the mature form of these enzymes reveals interesting features: murine submaxillary renin and porcine cathepsin D²⁰ possess two chains; in the case of renin the processing of these two chains occurs after a dibasic peptide. This activation cleavage is distinctly different from the intramolecular activation mechanism of pepsinogen²¹ but similar to that for proinsulin and other endocrine peptides²². It is not known whether submaxillary gland and kidney renins are identically processed from the same precursor molecule.

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Prostatic steroid binding protein: gene duplication and steroid binding

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The principal secretory protein in rat prostatic fluid is a steroid binding protein^{1–6} whose expression is stimulated by androgenic steroids^{7,8}. This prostatic steroid binding protein is oligomeric, consisting of two subunits, one containing the polypeptides C1 and C3 and the other containing C2 and C3⁹. We have isolated cDNA clones specific for each of the mRNAs which code for prostatic binding protein and note here the considerable DNA sequence homology between C1 and C2. Therefore, it is likely that duplication of an ancestral gene occurred and the subsequent divergence results in the expression of two related polypeptides that form part of a single protein. Furthermore, in view of the structure of the protein it is possible that the divergence of one of the genes has resulted in the capacity of this prostatic protein to bind steroids.

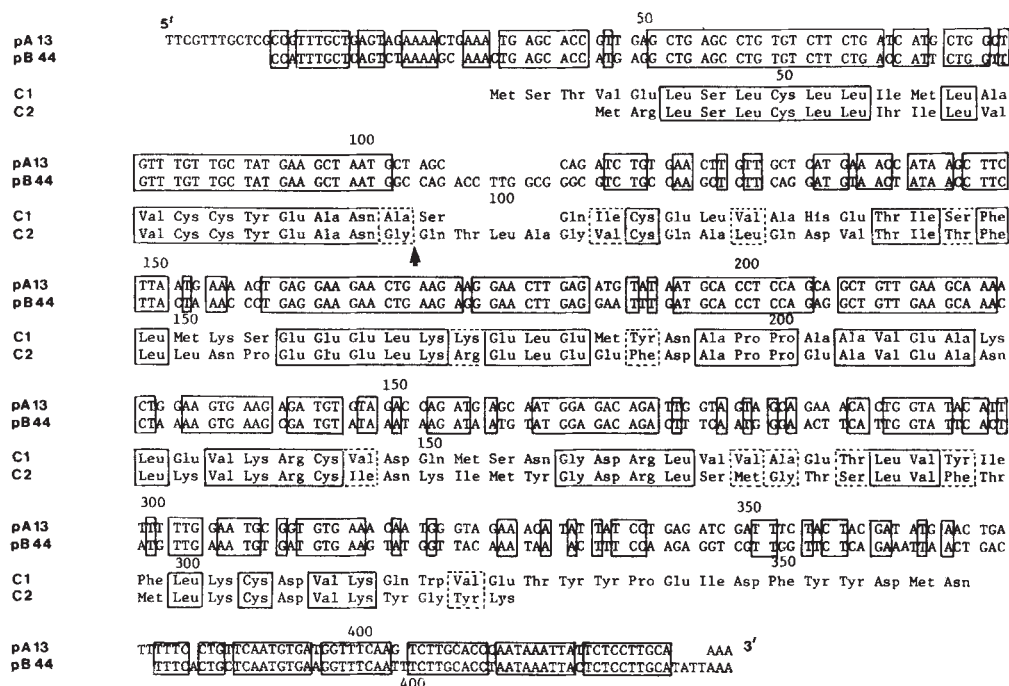
Prostatic steroid binding protein is an oligomer consisting of two subunits⁹. In one subunit C3, a polypeptide of molecular weight (M_r) 12,000 is linked by disulphide bridges to C2, a polypeptide of M_r 10,000 and in the other subunit C3 is linked by disulphide bridges to C1, a polypeptide of M_r 8,000. These three polypeptides are encoded by discrete mRNAs and we have previously isolated cDNA clones for C1 and C3 mRNA¹⁰ by using the ability of single-stranded cDNA to form a hairpin at its 3' end in order to prime the synthesis of the second DNA strand¹¹. As the cDNA inserts were much smaller than the estimated mRNA sizes, we have now used an alternative procedure which reduced the loss of DNA corresponding to the 5' end of the mRNA^{12,13}. Single-stranded cDNA was tailed with dCMP and synthesis of the second strand was primed with oligo(dG), thereby avoiding the use of S_1 nuclease which necessarily results in loss of DNA. After a second tailing reaction, dC-tailed double-stranded cDNA was inserted into the *Pst*I site of pAT153¹⁴, which had been tailed with dGMP.

Transformants that contained the desired cDNA sequences were identified by *in situ* hybridization¹⁵ using ³²P-labelled

Table 1 Relationships of prostatic binding proteins, mRNA and cDNA clones

Prostatic binding protein	Primary translation product	mRNA size (nucleotides)	cDNA plasmid	cDNA insert size (nucleotides)
8,000 (C1)	9,000	500–550	pA13	500
10,000 (C2)	11,000	550–600	pB44	500
12,000 (C3)	10,000	650–700	pA34	600

Fig. 1 Nucleotide sequence of prostatic binding protein mRNAs for C1 and C2 and deduced amino acid sequence. The nucleotide numbers are shown above the sequence. Gaps have been left to maximize homology; the solid boxes show perfect homologies and the dotted boxes show semi-conservative amino acid homologies. The putative cleavage point of the signal peptide is represented by $\uparrow$. The cDNA clones pA13 and pB44 which were used to analyse the sequences of C1 and C2 were isolated as follows. Poly(A)-containing RNA was isolated from rat ventral prostate and used to synthesize prostate cDNA⁸. The cDNA was 'tailed' using terminal transferase with dCTP in the presence of 1 mM CoCl₂ to yield molecules with ~20 dC at their 3' ends¹⁰. After phenol extraction the tailed DNA was passed through a Sephadex G-150 column and annealed with oligo(dG) to prime the synthesis of the second DNA strand using reverse transcriptase¹³. After phenol extraction and gel filtration the double-stranded cDNA was tailed again with dCTP using terminal transferase. pAT153¹⁴ was digested with *Pst*I and tailed with ~20 dGMP molecules. Annealing of pAT153 and double-stranded DNA was carried out at a molar ratio of 1:1 by heating the mix at 60 °C for 2 h and cooling overnight. After transformation of *Escherichia coli* HB101 had been carried out, recombinant colonies were selected for tetracycline resistance. Further screening to distinguish colonies which contained the desired prostatic DNA sequences was carried out by *in situ* hybridization using ³²P-labelled cDNA¹⁵. Plasmid DNA was isolated from transformed *E. coli* by lysis with lysozyme and subsequent SDS treatment in alkaline conditions³⁴. After purification on CsCl gradients the DNA was bound to diazobenzyloxymethyl paper as described previously¹⁶ except that 25 μ M sodium phosphate pH 6.5 was used in place of borate as buffer. The recombinant plasmid clones were then identified by mRNA purification. Total prostate poly(A)-containing RNA (6 μ g) was hybridized to the filters, washed and the bound RNA eluted and translated in a cell-free system derived from wheat germ³⁵. End-labelling of DNA with [γ -³²P]ATP and DNA sequence analysis were carried out using the methods of Maxam and Gilbert¹⁵. The sequencing strategy was to 5'-end label pA13 that had been digested with *Bgl*III and *Hind*III and pB44 that had been digested with *Hinf*I and *Hpa*II using [γ -³²P]ATP and T4 polynucleotide kinase.



cDNA. This was possible because the amount of hybridization with recombinant plasmids depends on the abundance of the individual cDNA species and the cDNAs that correspond to prostatic steroid binding protein mRNA represent by far the most abundant cDNA in the total mix. Individual recombinant plasmids were distinguished by mRNA purification^{10,16}. Thus cDNA clones pA13, pB44 and pA34 (Table 1), which are specific for C1, C2 and C3, respectively, were selected for further studies. It was likely that they represented complete copies of the mRNAs because we and others^{10,17,18} have estimated that the size of prostatic binding protein mRNAs, including the poly(A)-tail, was 450–700 nucleotides and the DNA insert sizes were consistent with the size of each corresponding mRNA (Table 1). Furthermore, preliminary mapping of mRNA transcripts synthesized *in vivo* indicates that the cDNA inserts extended to within 10 nucleotides of the 5' ends of the mRNAs.

We sequenced the cDNA inserts¹⁹ and found marked similarities between pA13 and pB44, resulting in extensive amino acid sequence homologies between C1 and C2 (Fig. 1). In contrast, the DNA sequence of pA34, which was consistent with the published protein sequence of C3²⁰, shows no significant homology with pA13 or pB44.

Both C1 and C2 mRNAs contain approximately 430 nucleotides. C1 mRNA contains: (i) A 5' non-coding region of at least 36 nucleotides. (ii) A coding region of 333 nucleotides of which 264 correspond precisely to the secreted polypeptide of 88 amino acids²¹. In fact, there are two potential AUG initiation codons which could represent the NH₂ terminus of the protein. As these would result in signal peptides of 12 or 23 amino acids, we favour initiation of translation at nucleotide 37 because all signal peptides described so far contain 15–30 amino acids²² and, in general, the first AUG initiation codon is utilized. (iii) A 3' non-coding region of 60 nucleotides, including a UGA termination codon and a AAUAAA poly(A) addition signal 19 nucleotides preceding the poly(A)-tail. The C2 mRNA contains: (i) A 5' non-coding region of 34 nucleotides. (ii) A postulated coding region of 294 nucleotides of which 60 probably comprise a signal peptides and 234 code for the secreted

polypeptide, in agreement with the partial protein sequence which has been determined to date (B. Peeters and W. Rombaux, personal communication). This would be consistent with the sizes of the primary translation product synthesized *in vitro* and the processed polypeptide secreted *in vivo*⁸. In addition, we suggest that the signal peptide is cleaved at Asn-Gly- $\uparrow$ -Gln which is used commonly by signal peptidases²². (iii) A 3' non-coding region of 105 nucleotides which contains a UAA termination codon and a AAUAAA poly(A) addition signal 24 nucleotides preceding the poly(A)-tail.

The most remarkable feature of the two mRNAs, however, is the extent of sequence homology which occurs in both coding and non-coding regions and which results in a 50% amino acid sequence homology between the primary translation products and increases to 62% when semi-conservative amino acids are included (Fig. 1). The most striking homologies are shown in Fig. 2 and encompass: (i) the 5' non-coding region of nucleotides 1–19 in pB44 and 13–31 in pA13 which share 79% homology, (ii) the signal peptide of nucleotides 22–99 in pB44 and 35–109 in pA13 which share 87% homology, (iii) the coding region of nucleotides 128–267 in pB44 and 131–269 in pA13 which share 76% homology and (iv) the 3' non-coding region of

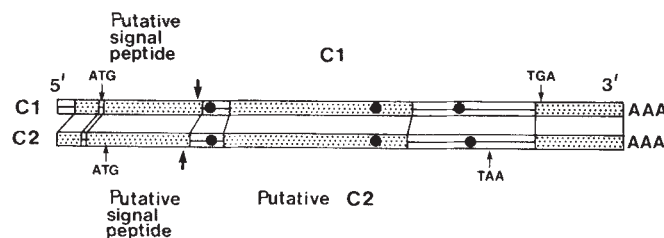


Fig. 2 Schematic representation of the major homologies between C1 and C2 mRNA. Hatched areas show regions of at least 75% DNA sequence homology. Possible homologous regions of less than 15 nucleotides have been omitted. The initiation codons ATG and termination codons TGA and TAA are shown by arrows and the putative signal peptides are shown preceding the arrows $\uparrow$ at which a signal peptidase may cleave. Cysteine residues (●) attach C1 and C2 to C3 by disulphide bridges.

nucleotides 362–432 in pB44 and 363–431 pA13 which share 81% homology. Several additional homologous regions of 15–20 nucleotides also exist but have been omitted for reasons of clarity.

The similarity in the DNA sequences in pA13 and pB44 suggest that the genes coding for C1 and C2 have probably arisen by duplication of an ancestral gene. There are now many examples of duplicated genes: some are pseudogenes^{23–25}, some are expressed at quite different levels with distinct control signals^{26–28}, and others are expressed in similar amounts^{29,30}. C1 and C2 seem to fall into this last class, being similarly expressed and controlled by androgens in the rat ventral prostate and ultimately both forming part of a secreted steroid binding protein. It is also of interest that in blotting experiments³¹ with rat and mouse DNA samples we find that pB44, but not pA13, hybridizes (at low as well as high stringency) with mouse DNA; therefore, we presume that gene duplication in the rat occurred after the evolutionary divergence of rat and mouse.

The divergence between the C1 and C2 genes may be of functional significance in view of the structure of prostate binding protein⁹ and its steroid binding properties^{2,6,32}. After dissociation of the protein into the two subunits, only C2–C3 continue to show steroid binding⁹. Although this experiment is open to several interpretations, the only apparent difference between the subunits is the composition of C1 and C2 and therefore one intriguing possibility is that a portion of C2 is crucial for steroid binding. In spite of this, it is extremely difficult to predict which regions of C2 are important because amino acid differences, which could markedly affect the conformation of the protein, occur throughout the two polypeptides. Moreover, it is conceivable that C2 does not bind steroids directly but may interact with C3 in such a way as to enable the C2–C3 subunit to bind steroids. Nevertheless, as estramustine, a nitrogen mustard derivative of oestradiol-17 β , binds with a K_d of 2×10^{-8} M³² and has been used as a therapeutic agent in the treatment of prostatic carcinoma³³, it will be of considerable interest to investigate whether similar genes can be detected in human DNA.

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Inverted repeats surround the ribitol-arabitol genes of *E. coli* C

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Only 10–20% of natural *Escherichia coli* strains can catabolize the pentitol sugars ribitol and D-arabitol¹. This variability extends to common laboratory strains: *E. coli* C strains, but neither K12 nor B strains, can catabolize these sugars. In *E. coli* C, the genes responsible for these catabolic pathways are chromosomal and consist of two very closely linked, independent operons². Genetic¹ and hybridization studies (C.D.L. and A.M.R., unpublished data) indicate that the genes are completely absent in K12. These observations led us to consider whether these genes had been acquired by horizontal, rather than vertical, genetic transmission, perhaps as an inserted transposable element. Here we report that the ribitol-arabitol genes of *E. coli* C are surrounded by 1.4 kilobase (kb) inverted repeats of imperfect homology. These genes may constitute an example of a vestigial transposable element recently acquired by the bacterial chromosome.

To examine the physical structure of the ribitol-arabitol chromosomal region, the ribitol-arabitol genes of *E. coli* C were cloned *in vivo* on a λ specialized-transducing phage (designated λ dAR) and *in vitro* by recombinant DNA techniques. Preliminary electron microscopy with DNA from λ dAR suggested that this phage contained inverted repeats, structures often associated with transposable elements^{3,4}. To investigate this possibility further, single strands of λ dAR and of two λ control phages were separated and purified by the poly(U, G)/CsCl equilibrium gradient method⁵. Renaturation of single-stranded phage DNA in the absence of its complementary strand yields duplex regions only by formation of intra-strand duplexes resulting from inverted repeat sequences in the original double-stranded molecule. These intrastrand duplex regions can be visualized and sized by degrading the unpaired single-stranded DNA with nuclease S₁ and displaying the remaining duplex DNA by gel electrophoresis⁶.

The gel banding pattern produced when single-stranded DNA from λ dAR and control phage λ Y199::Tn10 (which contains transposon Tn10) is treated with S₁ nuclease is shown in Fig. 1. Single-stranded λ dAR DNA produces multiple bands with a major band having the mobility of an ~1.4 kb fragment, and minor bands running at ~0.7 and 0.6 kb (Fig. 1, lanes f–i). These bands are never observed with single-stranded DNA from Y199, the parent phage of λ dAR. In contrast, DNA from phage λ Y199::Tn10 produces a single sharp band (Fig. 1, lanes b–e) corresponding to 1.36 kb, which is the size of the Tn10 inverted repeats as estimated by electron microscopy⁷. These results demonstrate that λ dAR, like λ Y199::Tn10, contains inverted repeats. The multiple banding pattern of λ dAR presumably reflects imperfect homology between two sequences of ~1.4 kb, resulting in single-strand 'bubbles' of non-homology in the intrastrand duplex which may be cleaved by S₁ nuclease. This view is supported by the observation that increasing the S₁ nuclease concentration shifts the banding pattern of λ dAR towards smaller, more numerous fragments (Fig. 1), and by hybridization experiments described below.

To locate these imperfect repeats with respect to the ribitol-arabitol genes, and to exclude the possibility that the inverted repeats found on the λ dAR genome are an artefact generated during *in vivo* formation of this phage, we also worked with the genes cloned by recombinant DNA techniques. Cloning was achieved by randomly ligating *Eco*RI fragments of *E. coli* C chromosomal DNA into the plasmid vector pBR322. (*Eco*RI was chosen after preliminary experiments demonstrated that an *Eco*RI fragment of λ dAR includes both the ribitol-arabitol

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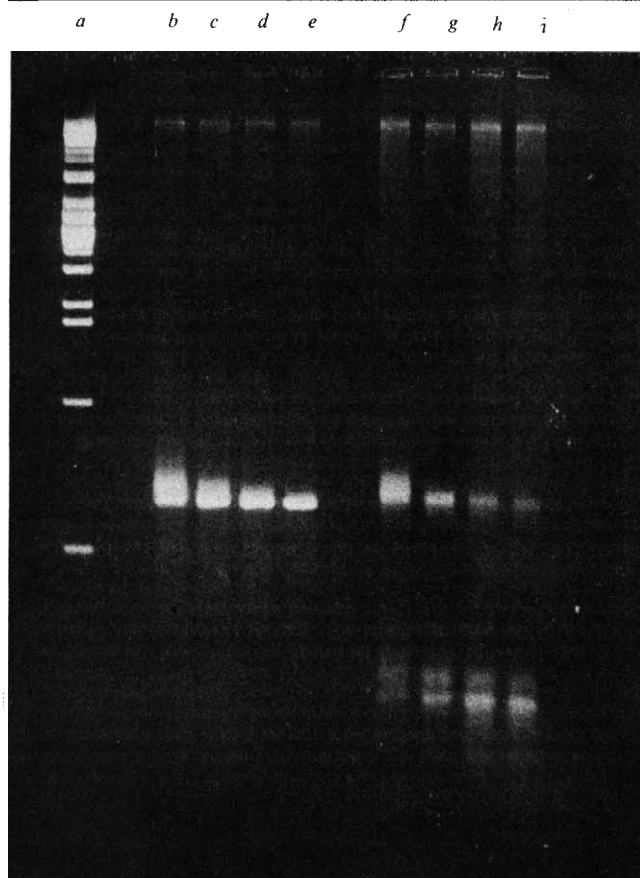


Fig. 1 Gel electrophoresis of intrastrand duplex fragments which survive S_1 nuclease digestion of renatured single-stranded DNA from λ Y199::Tn10 (lanes b-e) and λ dAR (f-i). Phage λ Y199::Tn10, used as a control, was constructed by selecting a recombinant between λ Y199 and the Tn10-containing phage λ 561¹⁸. Preparations of separated strands of phage DNA were renatured by heating for 1 h in 0.3 M NaCl at 65 °C. S_1 nuclease digestions were done as described earlier⁶ and the digested samples were run on a 1.4% agarose gel. Lane a contains an *Eco*RI-*Bam*HI double digest of λ^+ and pBR322 as a size standard. Lanes b-e contain 1.4 μ g of λ Y199::Tn10 DNA, f-i contain 3.2 μ g of λ dAR DNA. The digestions were done using 20 (b, f), 40 (c, g), 80 (d, h) or 120 (e, i) units of S_1 .

genes and the inverted repeats.) All transformants selected for their ability to use ribitol or D-arabitol could utilize both sugars, and all examined further harboured a plasmid in which a large (16.5 kb) *Eco*RI fragment had been inserted into pBR322. One of these plasmids, designated pAR-2, was chosen for further restriction mapping.

The relative locations of the inverted repeat sequences were determined by preparing Southern transfers of restriction enzyme digests of pAR-2 and probing with ³²P-labelled inverted-repeat DNA. The probe was prepared by first extracting from an agarose gel the major intrastrand duplex fragment which survives S_1 nuclease treatment of λ dAR (Fig. 1) and then labelling the fragment *in vitro* by nick translation⁸. Lane a of Fig. 2 is a positive control showing hybridization of the probe to the intrastrand duplex fragments produced by S_1 treatment of λ dAR. (The hybridization of the probe to the minor bands demonstrates that the smaller fragments are derived from the major intrastrand duplex fragment.) The hybridization patterns of the pAR-2 digests (Fig. 2b-e) were used to locate the inverted repeats relative to previously mapped restriction fragments of this plasmid. The repeats were found to lie in two non-contiguous fragments separated by 7.2 kb (see Fig. 3).

Similar hybridization studies show that the inverted repeat probe does not hybridize to Tn5, Tn10, or *E. coli* K12 chromosomal DNA, even in conditions that would detect partially homologous sequences⁹. Hybridization of the probe to *E. coli*

C chromosomal DNA was limited to fragments present in pAR-2, demonstrating that the only copies of the repeat unit in the *E. coli* C chromosome are those surrounding the ribitol-arabitol genes.

The location of the individual genes of the ribitol and D-arabitol operons relative to the inverted repeats was determined by experiments with pAR-2 plasmids into which the kanamycin-resistance transposon Tn5 had been inserted¹⁰. Inserts which prevented either ribitol or D-arabitol use were mapped by restriction enzyme analysis, and the individual genes inactivated were determined by genetic complementation. The gene order determined in this manner was in complete agreement with the order previously determined by standard genetic methods². The repeats bound the ribitol-arabitol genes in a transposon-like arrangement (Fig. 3). Although these experiments do not exclude the possibility that distal portions of the ribitol or D-arabitol genes contribute to the repeated sequences, results with the ribitol-arabitol region of the related bacteria *Klebsiella* have shown that no detectable hybridization exists between these operons, although hybridizing fragments were detected outside the operons¹¹.

As the repeat sequences surrounding the ribitol-arabitol genes are not found elsewhere in the *E. coli* C chromosome, or anywhere in the *E. coli* K12 chromosome, it is unlikely that they represent insertion sequences which coincidentally surround the ribitol-arabitol genes. The inverted repeats could result from residual homology retained from an ancestral tandem inverted duplication¹²; this is improbable, however, in light of the non-homology of the ribitol and D-arabitol operons. We

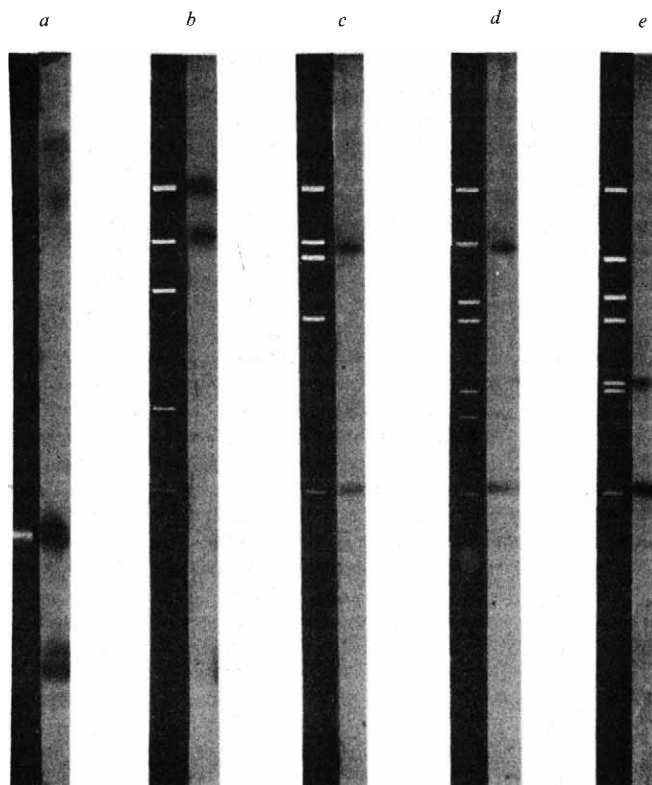
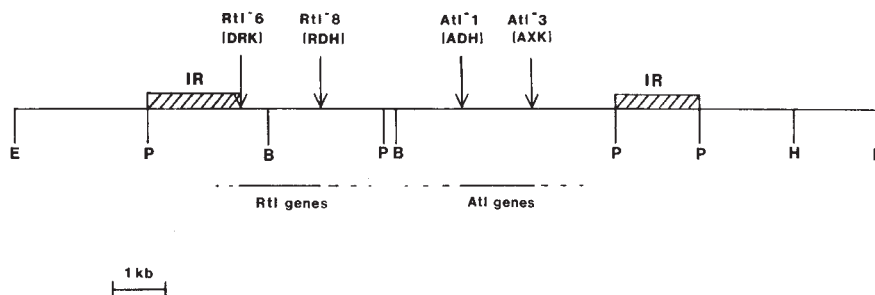


Fig. 2 Hybridization of the ribitol-arabitol inverted repeat probe to digests of pAR-2. The probe was created by electroeluting from an agarose gel the major intrastrand duplex fragment of λ dAR (see Fig. 1) and then labelling this fragment with ³²P by nick translation⁸. Restriction fragments of pAR-2 and pAR-2 derivatives were fractionated on a 1% agarose gel and transferred to nitrocellulose by the method of Southern¹⁹. Pretreatment of nitrocellulose filters and hybridization incubations were done as described previously²⁰. The probe was hybridized to: a, intrastrand duplex fragments generated by S_1 nuclease digestion of λ dAR single-stranded DNA; b, *Eco*RI-*Bam*HI-*Hind*III triple digest of pAR-2; c, *Pst*I digest of pAR-2; d, *Pst*I digest of pAR-2 containing a Tn5 insert in the gene for D-xylulose kinase; e, *Pst*I digest of pAR-2 containing a Tn5 insert in the gene for D-ribulose kinase.

Fig. 3 Map of the 16.5 kb *EcoRI* fragment cloned in pAR-2. Hatched regions indicate fragments which hybridize to the inverted repeat (IR) probe. Arrows indicate location of representative Tn5 insertions which inactivate ribitol or D-arabitol genes. Tn5 insertions were generated by delivering the Tn5 element via defective λ phage¹⁰ to strain AB1157 containing pAR-2. The subpopulation of pAR-2 plasmids which received Tn5 inserts was enriched by preparing plasmid DNA from all transposon-receiving cells, then using this to transform another strain to kanamycin resistance. Those inserts in pAR-2 were identified by screening the kanamycin-resistant transformants for inability to use ribitol or D-arabitol. Complementation studies were done by transforming a strain containing active ribitol and D-arabitol dehydrogenases, but inactive D-ribulose and D-xylulose kinases, with insert-containing plasmids. Because of the polarity of Tn5 insertions, this complementation test cannot distinguish between dehydrogenase and promoter insertions. Letters in parentheses indicate enzyme coded for by inactivated gene: DRK, D-ribulose kinase; RDH, ribitol dehydrogenase; ADH, D-arabitol dehydrogenase; AXK, arabitol-induced D-xylulose kinase. The maximum rightward extent of the left IR region was determined by experiments with pAR-2 containing Tn5 insertion Rtl⁻6, which show that only the left-hand plasmid-Tn5 junction fragment hybridizes to the probe (see Fig. 2, lane e). Restriction sites for *Sat*I, *Hpa*I and *Bgl*II were omitted for clarity. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I.



suggest that the inverted repeats are the structural remnants of an ancestral transposable element which carried the ribitol-arabitol genes into *E. coli*. This would account for the observation that only a subpopulation of *E. coli* strains can use ribitol and D-arabitol.

The imperfect homology of the inverted repeats suggests that they no longer function in transposition. Conjugation experiments (not shown) which demonstrate that the ribitol-arabitol genes do not seem to translocate from the chromosome ($<10^{-9}$ transpositions per gene copy) support this view. The hypothesis that the inverted repeats are derived from a transposable element could be further supported by demonstrating that the repeats themselves are flanked by short inverted repeats, or that the ends of the presumptive ribitol-arabitol transposon are flanked by a duplication of an oligonucleotide of target DNA. However, these transposon characteristics⁴ may be difficult to detect in this case because of the genetic drift which has apparently occurred since the insertion of these genes.

If the *E. coli* ribitol-arabitol region did originate as a transposon, then these genes can be viewed as a rare example of genes for which there is a molecular record of their acquisition by a bacterial chromosome¹³. What other chromosomal genes might have been derived from transposable elements? As transposons seem to carry genes of potentially strong selective advantage, chromosomal genes conferring catabolic traits or resistance to toxic agents could be derived from transposable elements. For example, certain chromosomal genes which confer antibiotic resistance (such as *ampC* of *E. coli*¹⁴) might also be found to be surrounded by inverted repeats. The *E. coli* genes for restriction and modification (*hds*), which are non-essential and can convey potentially strong selective advantage, may also be candidates for genes derived from transposons. (Restriction and modification genes have not yet been identified as part of transposable elements, although they can be phage or plasmid borne¹⁵.) Some of the inverted repeat sequences detected in the *E. coli* chromosome by electron microscopy^{16,17} may be evidence of past transposon insertions. The fraction of chromosomal genes derived from transposons may be significantly greater than the fraction of genes eventually found associated with inverted repeats, as one would expect the identifying structural features of genes derived from transposons to be lost with time.

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LexA protein inhibits transcription of the *E. coli* *uvrA* gene *in vitro*

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Treatment of *Escherichia coli* with radiation or chemical carcinogens induces a number of cellular functions to counteract the damage inflicted by such treatments (see ref. 1 for a review). One of these inducible functions is the SOS response, which is controlled by the *recA* and *lexA* genes: single-stranded DNA produced as a result of DNA damage binds to *recA* protein (RecA), activating its protease function; activated RecA cuts *lexA* protein (LexA), the repressor of the SOS genes, thereby inactivating it and turning on these genes²⁻⁴. The SOS genes include *recA* and *lexA* themselves, and recent work has shown that the two genes have similar operators where LexA binds *in vitro*, inhibiting their transcription^{3,4}. It is anticipated that other genes under the control of *recA* and *lexA* will have similar operators. Recent genetic^{5,6} and biochemical⁷ data suggest that the excision repair genes *uvrA* and *uvrB* are part of the *recA*-*lexA* regulon and are at least partly responsible for *recA*-*lexA*-mediated inducible DNA repair. In support of these results we have reported that *uvrB* has a promoter that is repressed by LexA *in vitro*⁸. We now show that *uvrA* has an operator similar to those of other SOS genes and that LexA binds to this operator and inhibits the transcription of the *uvrA* gene *in vitro*.

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To study the regulation of *uvrA* we used the plasmid pDR1996 (see Fig. 1a) which carries both the *uvrA* and *ssb* genes⁹. The exact location of *ssb* on this plasmid was determined from DNA and protein sequencing⁹. The direction of transcription of *uvrA* and the approximate location of its promoter were determined by Tn1000 mapping¹⁰. The exact location of the *uvrA* promoter was determined by subcloning and transcription experiments: removal of the *Bal*I–*Kpn*I fragment spanning 6.7–7.5 kilobases (kb) inactivated *uvrA* while transcriptional experiments indicated a single transcriptional start between the *Hae*III site at 6.73 kb and the *Hinf*I site at 7.03 kb (data not shown). This transcript was presumed to be that of *uvrA*, and this (*Hae*III–*Hinf*I)₃₂₅ fragment was sequenced to obtain the nucleotide sequence of the *uvrA* promoter shown in Fig. 1b. The transcriptional start point in this sequence was determined from RNA sequencing of the [γ -³²P]-labelled transcript made *in vitro* (data not shown) and is indicated as +1 in the figure.

To study the effect of LexA on *uvrA* transcription, LexA was included in the *in vitro* transcription reactions. As seen in Fig. 2, when present at concentrations similar to those used for *recA* and *lexA*², LexA markedly inhibits transcription of the *uvrA* gene. This inhibition is the result of specific binding of LexA to the promoter-operator region of *uvrA*, as is demon-

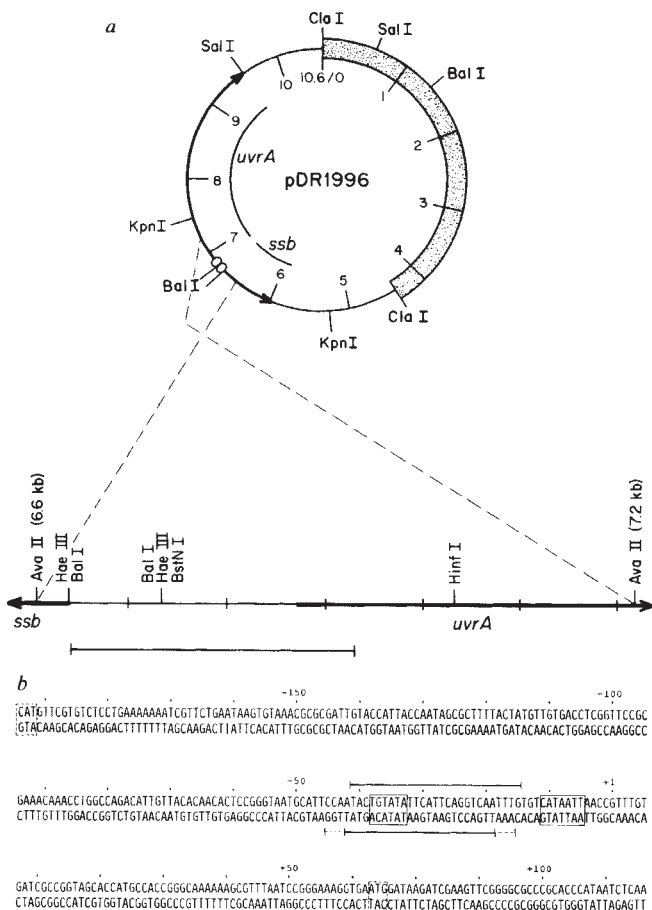


Fig. 1 a, Restriction map of the *uvrA* plasmid, pDR1996. The vector (pBR322) sequences are shaded. The locations and directions of transcription of *uvrA* and *ssb* are indicated by the heavy arrows on the map. The region whose sequence is shown in b is indicated by a bar beneath the detailed restriction map of the 0.6-kb *Ava*II fragment carrying the *uvrA* and *ssb* promoters. b, Sequence of the promoter-operator regions of *uvrA* and *ssb*. The -35 sequence and Pribnow box for *uvrA* are boxed. The previously determined⁹ initiation codon of *ssb* and the initiation codon for *uvrA* (unpublished data) are indicated by dashed boxes. The region protected from DNase I by LexA is bracketed; dashed lines at the ends of the brackets indicate regions of uncertainty as to the extent of LexA protection. Numbering is from the transcription start site of *uvrA* determined by RNA sequence analysis as previously described⁸.

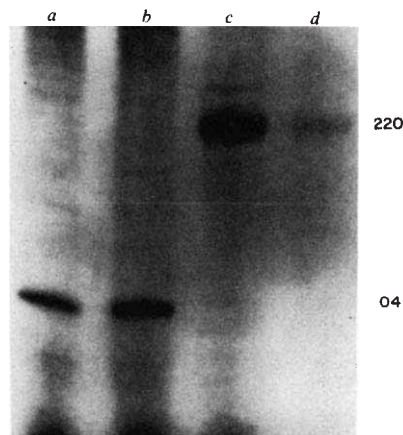


Fig. 2 Inhibition of *uvrA* transcription by LexA *in vitro*. pBR322 DNA and the (*Hae*III–*Hinf*I)₃₂₅ fragment of pDR1996 were transcribed *in vitro* in the presence and absence of LexA. Transcription reactions using 2.5 μ g ml⁻¹ of template and 14 μ g ml⁻¹ of LexA were performed as described previously⁸ except that the transcription buffer contained 30 mM KCl. Transcripts were analysed on a 6% polyacrylamide gel containing 9 M urea and visualized by autoradiography. In lanes a and b 75% of the reaction mixture was loaded onto the gel while in lanes c and d 25% of the total reaction volume was loaded. The length of the transcript (shown in nucleotides at the right of the figure) was determined by comparison with ³²P-labelled Φ X174 DNA. The 104-nucleotide transcript from pBR322 (used here as a control) has been described previously by Levine and Rupp¹³. The 220 nucleotide-long transcript initiated from the *uvrA* promoter was found to consist of two discrete RNA species which differed in length by ~10 nucleotides but possessed the same sequence at their 5' ends (data not shown). a, pBR322 minus LexA; b, pBR322 plus LexA; c, pDR1996 (*Hae*III–*Hinf*I)₃₂₅ minus LexA; d, pDR1996 (*Hae*III–*Hinf*I)₃₂₅ plus LexA.

strated by the DNase I protection experiments ('footprinting', see Fig. 3). As seen in Fig. 3, LexA protects from DNase I digestion a 25–30-base pair (bp) segment that overlaps the *uvrA* promoter.

Inspection of the protected region reveals that it has extensive sequence homology with the LexA binding sites found in the *recA*, *lexA* and *uvrB* genes^{2,3,8} and the postulated LexA binding site in the colicin E1 gene, *cle1* (ref. 11). A comparison of these various operators is shown in Fig. 4, from which two conclusions can be reached. First, although all the operators have considerable sequence homology with the *recA* operator, which has a near-perfect dyad symmetry, the only conserved dyad symmetry is 5'CTG(N)₁₀CAG3', suggesting that this structure might be an important feature of the LexA binding site. Second, the positioning of the LexA binding site relative to the two promoter defining sequences, the '-35 sequence' and the Pribnow box, varies in the different genes. Thus, the LexA binding site overlaps with the -35 sequence of *uvrA*, occupies the region extending from the -35 sequences to the Pribnow boxes of *recA* and *uvrB*, covers the Pribnow box and the transcriptional start site of *lexA* and occupies the region 3' to the Pribnow box of *cle1*. These varied locations of the LexA binding site may provide flexibility for additional regulatory circuits for individual genes by leaving specific regions in the promoters accessible to regulatory factors other than LexA. In this regard, it is of interest that the *uvrA* and *uvrB* genes share a homologous sequence that is only partly included in the LexA binding site (see Fig. 4). This sequence may be the recognition site of a repressor specific for these genes. (Note that such a repressor would inhibit transcription from the first *uvrB* promoter, P1, which is not influenced by LexA *in vitro*⁸.) Similarly, there is evidence that two other *recA*–*lexA*-controlled genes, *cle1* and *himA*, have second repressors in addition to LexA^{11,12}, and it is likely that the operators for these repressors do not or only partly overlap with the LexA binding site. The SOS response is a drastic reaction by the cell, causing a major perturbation in cell physiology and in some cases, even leading to cell death

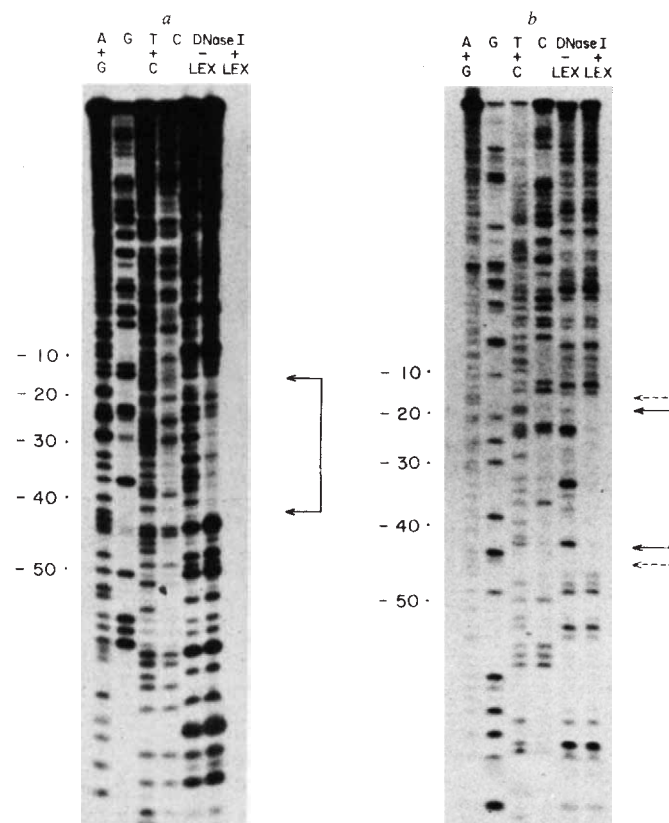


Fig. 3 Protection of the *uvrA* operator by LexA against digestion with DNase I (footprinting). The (*Hae*III-*Hinf*I)₃₂₅ fragment of pDR1996 was labelled with ³²P at the *Hae*III site at either the 5' or 3' end and then subjected to chemical sequencing¹⁴ or DNase digestion¹⁵ in the presence or absence of LexA. The conditions for footprinting were essentially as described before^{4,8,15}. The bases are numbered relative to the start point of *uvrA* mRNA, which is designated +1. The protected regions are bracketed and the positions where the effect of LexA protection could not be determined due to lack of DNase I cleavage are indicated by broken lines. *a*, Top strand (5' label); *b*, bottom strand (3' label).

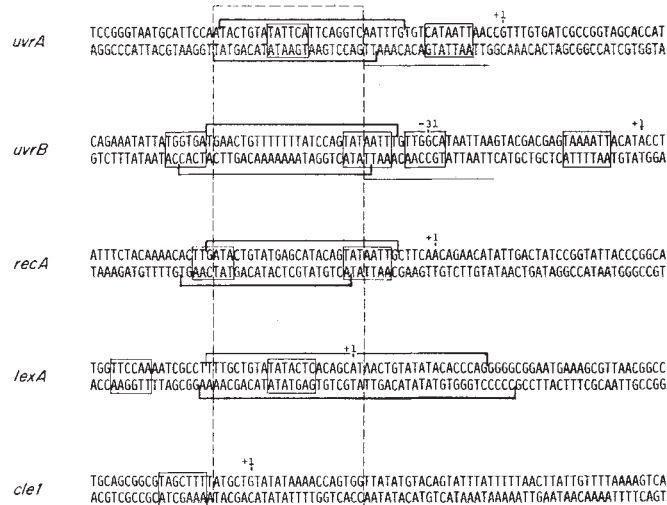


Fig. 4 Location of LexA binding sites in the promoter-operator regions of various genes that are controlled by *recA* and *lexA*. The 22-base pair dyad symmetry in the *recA* gene and the sequences in the other genes corresponding to this palindrome are in the dashed box. The -35 sequence of *cleI* (colicin E1) is not included for simplicity. Note that there are two tandem operators in *lexA*³ and that the potential LexA binding site of *cleI* is included in a larger dyad symmetry¹¹. *uvrB* has at least two transcriptional start sites, one marked +1 and the second at -31. Transcription from -31 but not from +1 is inhibited by LexA⁸. The regions of sequence homology between *uvrA* and *uvrB* are underlined. Other symbols are as in Fig. 1.

(as in the case of lambda induction). Thus, it may be advantageous for genes repressed by LexA to have additional regulatory controls that can function to fine-tune the response of different genes to different inducing stimuli, and perhaps also to cause expression of some SOS genes in conditions that do not lead to a full-scale SOS response.

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The *uvrD* gene of *E. coli* encodes a DNA-dependent ATPase

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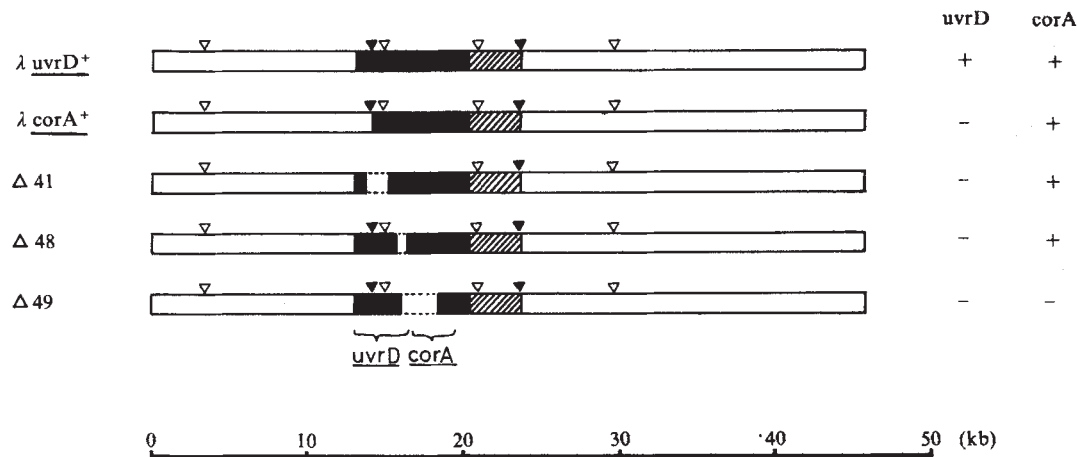
Mutations in the *uvrD* gene of *Escherichia coli* K-12 result in multiple phenotypes, including increased sensitivity to UV light and ionizing radiation, decreased ability for host cell reactivation, increased spontaneous mutation frequency and decreased rate of genetic recombination¹⁻⁶. It has been shown, moreover, that double mutants having mutations in both *uvrD* and *polA* genes are conditionally lethal⁷⁻¹⁰. The pleiotropic phenotypes of the *uvrD* mutants suggest that the *uvrD* gene product may have important roles in the processes of DNA repair, recombination and replication. To study the function of the *uvrD* gene, we have constructed hybrid phages and plasmids that carry the gene¹¹. Here we have analysed the proteins synthesized by the hybrid phages and report that the *uvrD* gene product is a polypeptide of molecular weight (MW) 75,000. We present evidence that the *uvrD* protein possesses a DNA-dependent ATPase activity.

A hybrid phage, λ uvrD⁺, which is able to complement *uvrD* mutations, was constructed using λ gt- λ c as cloning vector¹¹. In addition to the *uvrD* gene, λ uvrD⁺ carries the *corA* gene that controls transport of Mg²⁺, Mn²⁺ and Co²⁺ through the cell membrane^{12,13}. A hybrid phage, λ corA⁺, which carries the *corA* gene alone, was also constructed¹¹; this served as a control. We have isolated various deletion mutants from λ uvrD⁺ using the method of Saito and Uchida¹⁴. Of these deletion mutants, Δ 41 and Δ 48 were able to complement *corA* but not *uvrD* mutations, while Δ 49 was unable to complement either mutation.

λ uvrD⁺ contains a 6.8-kilobase pair (kbp) DNA fragment derived from the *E. coli* chromosome and a part (4.0 kbp) of the γ d sequence (transposon Tn1000) derived from F plasmid¹¹. DNA fragments generated from these phages using restriction endonucleases were analysed by agarose gel electrophoresis, and physical maps constructed (Fig. 1).

Proteins produced by the λ transducing phages were labelled with a ¹⁴C-amino acid mixture and analysed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Compared with cells infected with λ gt- λ c, two specific bands, corresponding to polypeptides

Fig. 1 Physical maps of transducing λ phages. Cleavage maps were constructed by digestion with restriction enzymes followed by agarose gel electrophoretic analysis as described previously¹¹. Origins and restriction enzyme target sites are: ■, *E. coli* DNA; ▨, $\gamma\delta$ sequence; □, λ phage DNA; [], deletion; ▽, *Bam*HI target sites; ▼, *Eco*RI target sites. Phenotypes of *uvrD3* or *corA5738* cells lysogenized with each transducing phage are shown on the right-hand side.



of molecular weight 75,000 and 37,000, were found in cells infected with λ *uvrD*⁺. The 75,000-MW polypeptide was identified as the *uvrD* gene product, because it was absent from cells infected with λ *corA*⁺, Δ 41 or Δ 48, which lack the intact *uvrD* gene. The 37,000-MW polypeptide seems to be the *corA* gene product; it is absent from Δ 49.

Of the many proteins known to be involved in DNA metabolism, there are some whose molecular weight is ~75,000. These include DNA-dependent ATPase I^{15,16} and DNA helicase II^{17,18}. Recent studies suggested that they may be the same proteins¹⁹. Thus, we have examined DNA-dependent ATPase activity in cells infected with *uvrD*⁺ transducing phage. To overproduce the *uvrD* protein, the *susQ* mutation²⁰ was introduced into both λ *uvrD*⁺ and λ *corA*⁺ by recombination of each phage strain with λ *imm434 susQ21*. A culture of *E. coli* W3623 (*uvrD*⁺ *Su*⁻) infected with λ *uvrD*⁺ *susQ* was collected 195 min after infection, and an extract was subjected to DEAE-cellulose filtration, ammonium sulphate fractionation and phosphocellulose chromatography. As a control, an extract of λ *corA*⁺ *susQ*-infected cells was processed similarly.

Figure 3 shows elution profiles of DNA-dependent ATPase activity from phosphocellulose columns. Both samples showed activity eluting as a single peak at almost the same position as that reported for DNA-dependent ATPase I in these conditions¹⁵. The ATPase activity was totally dependent on denatured DNA and was sensitive to *N*-ethylmaleimide, as was

observed with the enzyme from normal cells. We estimated that λ *uvrD*⁺-infected *uvrD*⁺ cells contain about 14 times more ATPase activity than λ *corA*⁺-infected *uvrD*⁺ cells. The small peaks in Fig. 3b may represent composites of ATPases, which were shown to be present in phosphocellulose eluates²¹.

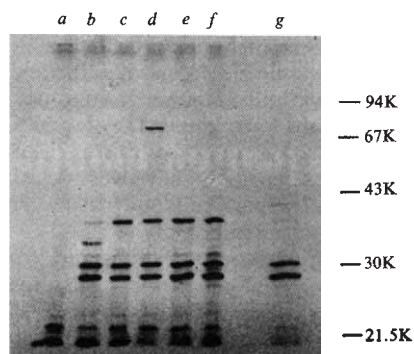


Fig. 2 Proteins produced by transducing λ phages. *E. coli* KY3304 lysogenic for λ C1857 was grown in RM medium at 30 °C to a density of 2×10^8 cells ml⁻¹ and infected with one of the transducing phages at a multiplicity of 10. The cells were labelled with ¹⁴C-amino acid mixture at 30 °C for 50 min and extracts were prepared²⁸. The polypeptides were analysed by electrophoresis on 10% SDS-polyacrylamide gels followed by fluorography, as described previously²⁹. The positions of phosphorylase B [MW 94,000 (94K)], bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K) and soybean trypsin inhibitor (21.5K) are indicated. a, Uninfected; b, λ gt $\cdot$ λ c; c, λ *corA*⁺; d, λ *uvrD*⁺; e, Δ 41; f, Δ 48; g, Δ 49.

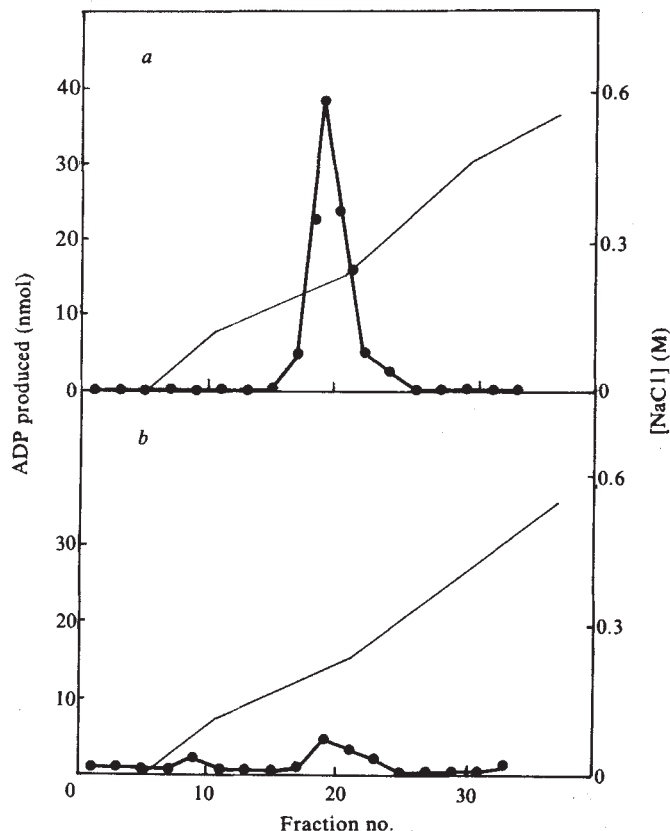


Fig. 3 Chromatographic resolution of DNA-dependent ATPase. *E. coli* strain W3623 (*uvrD*⁺ *Su*⁻) was infected with λ *uvrD*⁺ *susQ* (a) or λ *corA*⁺ *susQ* (b). The extracts were prepared from ~3 g of the infected cells and processed as described elsewhere¹⁵. Fraction II was applied to a phosphocellulose column (0.6 × 6 cm) and eluted with a linear gradient of 0.04–0.6 M NaCl in 0.02 M potassium phosphate buffer (pH 7.2). DNA-dependent ATPase activity was determined using 5 μ l of each fraction. The reaction mixture (75 μ l final volume) contained 50 mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol, 2 mM MgCl₂, 1 mM [γ -³²P]ATP (1×10^5 counts min⁻¹), 15 μ g bovine serum albumin, and 7.5 nmol of heat-denatured calf thymus DNA. Incubation time, 20 min. A peak fraction (no. 19) of each sample was used for further analysis (see Fig. 4).

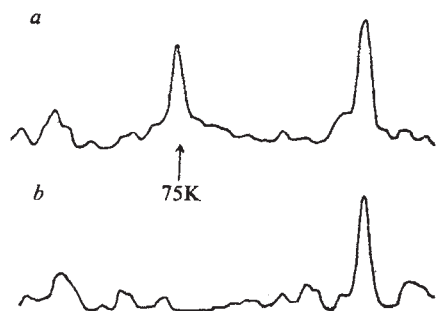


Fig. 4 Analysis of DNA-dependent ATPase fractions by SDS-polyacrylamide gel electrophoresis. Enzyme fractions (14 μ l) from λ uvrD⁺-infected (a) and λ corA⁺-infected (b) cells were applied to 10% SDS-polyacrylamide gels and electrophoresed at 20 mA for 3 h. The gels were stained with Coomassie brilliant blue and scanned using a densitometer. Electrophoresis was from right to left. An arrow indicates the 75,000-MW polypeptide.

The enzyme fractions were further analysed by SDS-polyacrylamide gel electrophoresis. Figure 4 shows the patterns of proteins present in each of the peak fractions. In the λ uvrD⁺ sample there was a large distinct band corresponding to the 75,000-MW protein whereas only a small band was observed at the corresponding position of the λ corA⁺ sample, although the sizes of the other bands were almost the same for both samples. The ratio of the amounts of the 75,000-MW protein for the two samples was 12:1, very similar to that of the activities of the two samples.

These results suggest that the *uvrD* gene encodes a 75,000-MW protein which possesses a DNA-dependent ATPase activity. As this protein is similar in both size and chromatographic properties to DNA-dependent ATPase I^{15,16}, it is possible that the *uvrD* protein may be identical to DNA-dependent ATPase I. Recently, Richet *et al.*¹⁹ isolated mutants that are defective in DNA-dependent ATPase I. Although they have correlated the mutations neither to certain phenotypes nor to a specific chromosomal locus, it is possible that the mutations might occur in the *uvrD* gene.

E. coli possesses several enzymes capable of unwinding DNA at the expense of ATP^{17,18,21-27}, and such enzymes may have vital roles in DNA replication, recombination and repair. Evidence has been presented that one of the enzymes, DNA helicase II, may be identical to DNA-dependent ATPase I¹⁹. Thus, it would be of interest to determine whether the *uvrD* protein possesses DNA unwinding activity. Studies on this are now in progress.

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A relationship between replicon size and supercoiled loop domains in the eukaryotic genome

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In interphase nuclei and metaphase chromosomes, eukaryotic chromosomal DNA is arranged in negatively supercoiled loops whose length averages several tens of kilobases (kb). Supercoiling indicates that the loops are tenaciously bound at both ends through the periodic attachment of DNA to a non-histone protein structural support, termed the nuclear matrix, skeleton or cage in interphase nuclei and the scaffold in metaphase chromosomes¹⁻¹⁶. The looped organization has been envisaged to be important in DNA replication^{2,11,15,17-25} and transcription²⁶⁻²⁸. We report here a close relationship between average loop length and replicon size in different animal and plant species. Autoradiographic experiments support the hypothesis that DNA synthesis occurs at fixed sites on the nuclear matrix^{15,20}. We also describe a modification of the looped organization occurring during early embryonic development in *Xenopus laevis*.

We have investigated the supercoiled loop organization of erythrocytes (Fig. 1) and cultured cells (not shown) from *X. laevis* using the fluorescence halo technique¹¹. Cells were treated with the non-ionic detergent NP40, dehistonized in 2 M NaCl containing various amounts of ethidium bromide or acridine orange and observed under the fluorescence microscope. The intercalation of either dye causes the dehistonized loops to form a DNase-sensitive and RNase-resistant fluorescence halo around the nuclear skeleton (Fig. 1b, d). The halo's radius (15 μ m maximum) exhibits a characteristic 'breathing' in response to varying dye concentrations and prolonged UV irradiation (see Fig. 1 legend and refs 11, 23), thus supporting conclusions from different systems^{11,23} that DNA is arranged in independent negatively supercoiled loops. Assuming that the largest fluorescence halo corresponds to completely unwound and extended DNA loops, then its radius gives a rough estimate of the average loop size (2 $\times$ 15 μ m or $\sim$ 90 kilobases in *X. laevis*).

We next investigated the relationship between the nuclear matrix, supercoiled loops and DNA replication in cultured *X. laevis* kidney cells using the fluorescence halo technique coupled with autoradiography. Asynchronous cells grown in monolayer on microscope slides were ³H-thymidine-pulsed for 2 min. The cells were then treated *in situ* for maximal halo expansion and autoradiographed. After 2 min labelling, radioactivity covers almost exclusively the nuclear matrix (Fig. 2a).

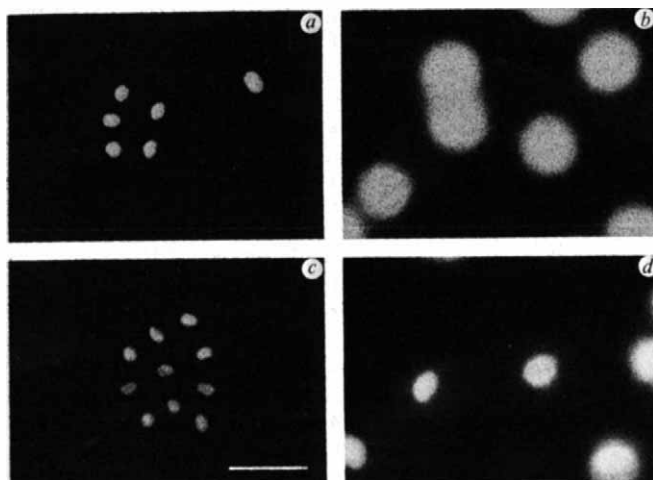
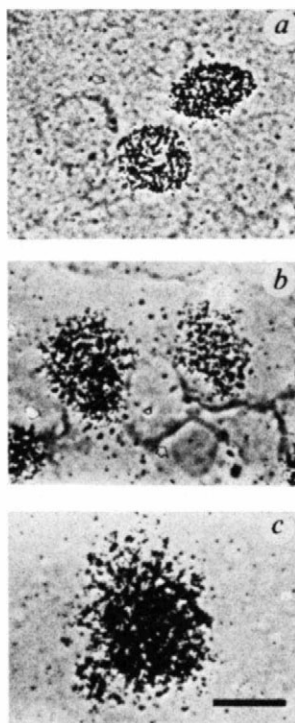


Fig. 1 Fluorescence micrograph of *X. laevis* erythrocyte nuclei. Freshly prepared blood smears were dipped for 30 s in ice-cold NP40 buffer (0.5% NP40, 10 mM MgCl₂, 0.5 mM CaCl₂, 1 mM phenylmethylsulphonyl fluoride, 50 mM Tris-HCl pH 7.5), stained with 5 μ g ml⁻¹ ethidium bromide (a) or 5 μ g ml⁻¹ acridine orange (c) and observed under the fluorescence microscope. When the dyes were dissolved in high-salt buffer (2 M NaCl, 0.2 mM MgCl₂, 10 mM Tris-HCl pH 7.5) at the same concentration a distinct fluorescence halo appeared (b, ethidium bromide; d, acridine orange). The halo radius (from the edge of the nuclear matrix to the edge of the halo) was measured using a micrometer eyepiece during the first few seconds following UV illumination. The radius reaches its maximum, ~15 μ m, when the fluorochrome concentration is ~5 μ g ml⁻¹; the lower or the higher the dye concentration, the smaller the halo (data not shown). However, at every dye concentration the maximal radius is attained following prolonged UV irradiation under the fluorescence microscope. These observations indicate that, with increasing dye concentrations, DNA loops are progressively unwound from a negatively supercoiled to a relaxed state and eventually forced into a positively supercoiled condition; whereas—independently from dye concentration—nicks introduced in the DNA by prolonged UV exposure remove the topological constraints thus lifting the maximal halo^{11,23}. Scale bar, 45 μ m.

Fig. 2 Autoradiographs of *X. laevis* kidney culture cells pulsed with ³H-thymidine and treated for maximal halo expansion. The fibroblast clone used stems from an established cell line (from Dr M. Cianfriglia, Siena). Cells were cultured in Eagle's minimal essential medium H₂O (100:40), supplemented with 10% fetal calf serum, at 26°C under 5% CO₂. Asynchronous cells growing in monolayer on microscope slides were pulsed with 20 μ Ci ml⁻¹ ³H-thymidine (42 Ci mmol⁻¹; Amersham) for 2 (a), 10 (b), 30 (c) and 60 min or, in pulse-chase experiments (not shown), for 2 min followed by 0, 8, 28, 58 min chases with a 100-fold excess cold thymidine. The slides were then sequentially dipped in ice-cold distilled water for a few seconds, ice-cold NP40 buffer for ~30 s followed by 100 μ g ml⁻¹ ethidium bromide in high-salt buffer (see Fig. 1 legend) for ~1 min and exposed to a UV lamp (15 W, short wave) at a distance (~5 cm) and for a time sufficient for maximal halo expansion. After briefly rinsing with distilled water the slides were air dried and autoradiographed using Kodak NTB 2 undiluted autoradiographic emulsion. Exposure time was varied to obtain a roughly comparable grain density. Smoothed contours of several labelled areas, and of several unlabelled nuclear matrixes, were traced on enlarged prints of the autoradiographs, and assimilated to a circumference, using a Tektronix 4956 digitizer coupled to a 4051 desk top computer. Mean radius (in μ m) of the labelled areas $\pm$ s.d. (number of measurements): 2 min pulse, 13.0 $\pm$ 0.85 (27); 10 min pulse, 17.2 $\pm$ 1.8 (30); 30 min pulse, 26.3 $\pm$ 1.9 (25); 60 min pulse, 27.6 $\pm$ 1.4 (29). Radius of the unlabelled nuclear matrix: 12.1 $\pm$ 0.9 μ m (30). Average expansion rate of the labelled area: 0.47 μ m min⁻¹. Arrows point to unlabelled nuclear matrixes. Scale bar, 25 μ m.



Considering that the cells were in asynchronous growth and active replicons were thus in all phases of replication, this observation suggests—in agreement with other investigations on different biological systems^{2,11,18,20-23}—that in *X. laevis*, DNA synthesis occurs at fixed sites on the nuclear matrix. Nevertheless, the possibility that newly synthesized DNA binds nonspecifically to the nuclear matrix during extraction cannot be ruled out. Following 10 and 30 min pulses, the radioactivity distributes on a progressively wider area, which extends beyond the nuclear matrix (Fig. 2b, c). The radioactive area expands at a rate of ~0.47 μ m min⁻¹ (see Fig. 2 legend), in agreement with estimates of fork movement rate from DNA fibre autoradiography²⁹. Almost identical results were obtained in pulse-chase experiments (data not shown) in which the expansion rate of the radioactive area was ~0.43 μ m min⁻¹. Taking account of the bidirectionality of DNA replication and the average loop size in *X. laevis*, one loop would be predicted to be duplicated in ~30 min; hence pulses longer than 30 min should not result in a further increase of the labelled area. This was confirmed after 60 min labelling, since there was no substantial difference in the size of the labelled area compared with after 30 min.

The expansion of radioactivity on the halo with increasing pulse length suggests that replicating DNA loops are reeled through matrix-bound replication complexes, as proposed by Pardoll *et al.*^{15,30}. On this interpretation, a relationship between the average loop size and the average replicon length would be predicted. We have chosen several animal and plant species

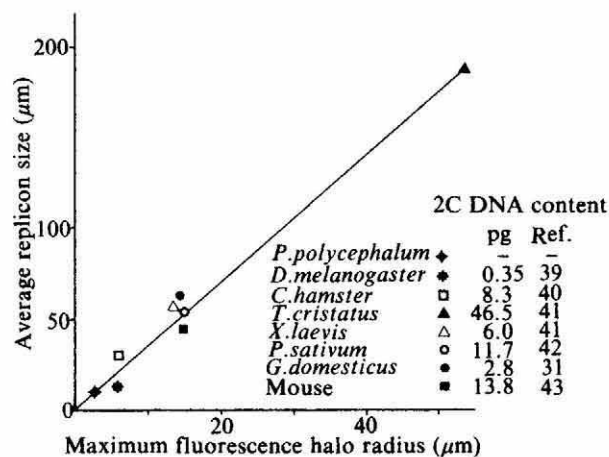


Fig. 3 Relationship between maximum fluorescence halo radius (MFHR) and average replicon size (av. RS). MFHR was determined by treating blood smears, cultured cells grown on microscope slides or isolated nuclei with ice-cold NP40 buffer and staining with 100 μ g ml⁻¹ ethidium bromide in high-salt buffer (see Fig. 1 legend). Maximal fluorescence halo expansion was induced by prolonged ($\geq$ 2 min) UV irradiation under the fluorescence microscope and measured using a micrometer eyepiece. Mean MFHR values (in μ m) $\pm$ s.d. (number of measurements) are given below. MFHR data for *P. polycephalum* and mouse and all av. RS values (in μ m) determined by DNA fibre autoradiography are taken from the literature. *P. polycephalum*: av. RS, 9³⁸; MFHR 2.5–3²³. *Drosophila melanogaster*: av. RS, on Schneider 2 cultured cells, 13.6³²; MFHR, on GM 2 cultured cells (gift of Dr R. Canova, Centro Acidi Nucleici, Rome) 6.0 $\pm$ 0.8 (25). Chinese hamster: av. RS, on B14FAF28 cultured cells, 30³³; MFHR, on CHEF 125 cultured cells (grown in Eagle's minimal essential medium at 37°C under 5% CO₂), 6.1 $\pm$ 0.7 (31). *X. laevis*: av. RS, on A6 cultured cells, 57.5³¹; MFHR, on kidney cultured cells (see Fig. 2 legend), 14.0 $\pm$ 3.2 (19). *Pisum sativum*: av. RS, on cells from root tip meristems, 54.7³⁴; MFHR, on nuclei from root tips (isolation procedure as described in Fig. 4 legend) 14.8 $\pm$ 1.5 (25). *Gallus domesticus*: av. RS, on embryonic primary cell culture, 63³⁵; MFHR, on freshly prepared blood smear, 14.1 $\pm$ 1.7 (15). Mouse: av. RS, on L929 cultured cells, 45.4³⁶; MFHR, on 3T3 cultured cells, 15¹¹. *Triturus cristatus*: av. RS, on liver primary cell culture, 188³¹; MFHR, on freshly prepared blood smear, 53.7 $\pm$ 4.2 (24). The 2C DNA contents in pg (ref. from which value is taken) for the various species examined are the following. *P. polycephalum* (◆), (—); *D. melanogaster* (★), 0.35³⁹; Chinese hamster (□), 8.3⁴⁰; *T. cristatus* (▲), 46.5⁴¹; *X. laevis* (△), 6.0⁴¹; *P. sativum* (○), 11.7⁴²; *G. domesticus* (●), 2.8³¹; mouse (■) 13.8⁴³.

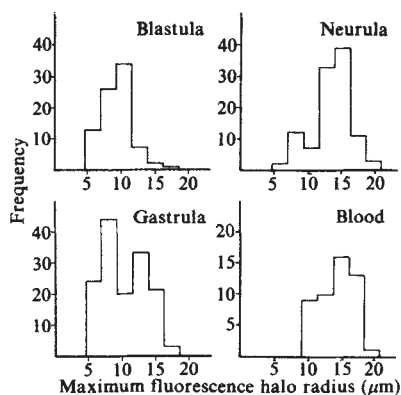


Fig. 4 Maximum fluorescence halo radius in different developmental stages of *X. laevis*. Ovulation and mating were stimulated by injection of gonadotropic hormone into the dorsal lymph sac a few hours before mating: females received 1,200 U of Pregnyl (Organon) and males 300 U. Mating and egg laying were allowed to occur overnight. The embryos were then collected, dejellied with 2.4% cysteine pH 7.5 and staged according to Nieuwkoop and Faber³⁷. Nuclei were isolated by gently homogenizing the embryos in buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM Tris-HCl pH 7.5) containing 0.34 M sucrose. One volume of ice-cold NP40 buffer (see Fig. 1 legend) was then added and the homogenate was centrifuged ($\sim 1,000g$ at 4 °C for 10 min) on a 1.37 M sucrose cushion in buffer A. The pellet was resuspended in a small volume of 20 mM EDTA, 10 mM Tris-HCl pH 7.5. A droplet of this suspension was diluted, directly on microscope slides, in an excess volume of 100 $\mu g\ ml^{-1}$ ethidium bromide in high-salt buffer (see Fig. 1 legend) and covered with a coverslip. Maximum fluorescence halo radii were measured under the fluorescence microscope, using a micrometer eyepiece, after prolonged (≥ 2 min) UV illumination. Blood smears were treated for maximal fluorescence halo expansion as described in Fig. 3 legend. Mean values (in μm) $\pm$ s.d. (number of measurements): blastula (stage 8–9), 9.4 ± 2.4 (83); gastrula (stage 10–11), 10.3 ± 3.3 (145); neurula (stage 13–14) 13.5 ± 3.0 (107); (adult blood) 14.5 ± 2.6 (49). The observation of fluorescent haloes on embryonic nuclei in suspension is quite difficult, as the DNA haloes, compared with those from blood smears or cells growing attached to glass surfaces (Fig. 3), can be easily distorted or destroyed by sudden currents or air bubbles in the liquid sheet between the microscope slide and the coverslip.

(Fig. 3) whose replicon sizes are known from DNA fibre autoradiography. The maximum halo radius seems to be species specific, varying from 2.5 μm (*Physarum polycephalum*) to 53.7 μm (*Triturus cristatus*), and is only roughly proportional to 2C DNA content (DNA content/diploid nucleus). On the other hand the halo radius (loop size) is directly proportional to the average replicon length, which strongly supports a general relationship between supercoiled loop structure and DNA replication.

It is of interest to speculate how DNA loops might be related to replication. A preliminary condition must be the existence of two adjacent matrix-bound replication complexes per replicon, in order to reconcile fixed replication sites with the bidirectionality of synthesis. As a further development of previous models^{2,15,19,20,22,30} we propose that each replicon might actually correspond to two adjacent loops and be duplicated by the reeling of the DNA through a couple of matrix-bound replication complexes, alternate anchorage sites of the DNA to the nuclear matrix behaving as origins and termini of replication. Once a new nuclear matrix is assembled—whenever this happens with respect to the S phase—one of the two newly replicated origins, and one of the two termini, would remain bound to the old matrix while the other would bind to the newly assembled one. This, admittedly speculative, view was drawn from the data in Fig. 3. The average replicon length in all the species explored is roughly four times the maximum fluorescence halo radius, that is, twice the size of a DNA loop. Although the replicon lengths and halo radii are only approximations, our interpretation is appealing because it suggests how the whole chromosome structure can be faithfully reproduced at each cell cycle and how daughter DNA strands orderly segregate at mitosis.

An intriguing facet of DNA replication in multicellular organisms is the brevity of the S phase in early, compared with later, development. As the rate of fork movement is constant, the variation has been explained on the basis that during pre-gastrular stages, compared with later ones, the origins of replication are synchronously activated and the replicon size is smaller^{31,32}.

To check further the proposed correspondence between replicons and supercoiled loops we have determined the maximum halo radius in embryonic cells from *X. laevis* at pre- and post-gastrular stages. The maximum halo size in blastulae averages 9 μm (Fig. 4). A significant proportion of gastrula-stage nuclei are able to develop haloes of 10–15 μm , and at the tail bud stage, most of the dehistonized nuclei have 10–15 μm haloes. These results are consistent with the reduced replicon size in pregastrular stages^{31,32} and further support a relationship between supercoiled loops and replication units¹¹. They suggest that structural rearrangement of the chromosome occurs during gastrulation, when the embryonic genome enters a phase of active gene expression. However, the intrinsic limitations of the technique used here (see Fig. 4 legend) require that this structural transition be confirmed by different experimental approaches. It is nonetheless tempting to envisage such a transition in gastrula as of crucial importance in establishing novel patterns of supercoiled loop domains representing both replication and transcription units.

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BOOK REVIEWS

Confronting the unanswerable

Marie Jahoda

WHEN two nimble minds combine in the effort to produce a book designed, according to the preface, "to provoke, disturb and befuddle its readers" the chances of succeeding in this not obviously laudable enterprise must be high. Had Hofstadter and Dennett added to their aims amuse, disappoint and frustrate they would have encompassed the whole universe of possible readers' reactions to this particular book and their success, measured by their own standards, would have been total.

The themes addressed in the book concern eternal questions that have preoccupied great and not so great minds since the dawn of history: the nature of self, of consciousness, of reality, the body-mind problem, free will, the relation of mechanism to meaning. Every conceivable approach to these issues has in the past been adopted with conviction by some; none has withstood critical examination by others. All of them, however, whether based on religion, philosophy or science fulfil a major function: they enable their adherents to engage in a bootstrap operation, pulling themselves out of the inevitable ignorance about the ultimate why and wherefore. Once this operation is performed they can proceed to tackle answerable questions or develop consistent world views.

The debate about fundamental assumptions has in recent years gained new impetus through the development of computers and of artificial intelligence (AI); both play a dominant part in the book under review. The issues raised are, however, also tackled by other means without recourse to modern technology or to the spreading enthusiasm for AI. This variety of approaches is the result of the peculiar conception of this book. Hofstadter and Dennett have here reprinted pieces by 20 different authors, each of whom is known as a science fiction writer or a philosopher or a scientist or a literary figure. If one did not know their names it would be hard to classify them, for many write with remarkable ease in several of these idioms. Every contribution is followed by some reflections on it by one or both of the "composers" of this volume. These original contributions form less than a quarter of the text.

Many, though not all of the reprinted pieces are amusing and relevant to the major issues. My favourite science fiction writer, philosopher and scientist, Stanislaw Lem, is represented by several pieces, outstanding among them *Non Serviam*, where his man-made creatures agonize about

The Mind's I: Fantasies and Reflections on Self and Soul. Composed and arranged by Douglas R. Hofstadter and Daniel C. Dennett. Pp.450. UK ISBN 0-7108-0352-4; US ISBN 0-465-04624-X. (Harvester/Basic Books: 1981.) £9.95, \$16.95.

their origin and the qualities of their creator; agonize because the very essence of their being precludes the possibility of discovering answers.

The philosopher Dennett in his excursion into science fiction raises the fantasy of a disembodied brain kept functioning while connected to an empty cranium by radio links, thus directing a human body hundreds of miles away. In his reflections on his own story Dennett rightly says that the story "not only isn't true . . . but couldn't be true" (p.230) and is truly "outrageous" (p.231). On p.5, however, he commits himself differently by insisting that everything that is imaginable is hence possible in principle; befuddling with a vengeance and disturbing in raising the possibility of brain transplants.

Richard Dawkins in an excerpt from *The Selfish Gene* is at his well-known reductionist best, but he admits that the evolution of subjective consciousness is "the most profound mystery facing modern biology" (p.141). Thomas Nagel's question, "What is it like to be a Bat?", prompts an interesting discussion of the gap between subjective experience and objective fact and the not-unexpected answer that it is hard to know.

In his reflection on this contribution Hofstadter, who has so brilliantly demonstrated his ability to discuss profound problems playfully and with wit in his *Prelude . . . Ant Fugue* (reprinted in this volume), here lets himself down by raising some 50 rather silly questions, of which "What is it like to be Chopin's brother [he had none]?" is a typical example. Nagel's question posed the problem; 50 variations do not make it clearer. To be sure, he follows this with other reflections, some of them interesting, but less coherent than Nagel's argument.

Computer simulation and AI appear in many original and reprinted contributions; they are confronted head-on in John Searle's "Minds, Brains and Programs" and in the following reflections. This sharp and prolonged controversy forms the frustrating highlight of the book. Searle distinguishes the cautious claim of AI — that it is a powerful tool for the study of the mind — from the strong claim that computers given the right programs can be

literally said to *understand* . . . the programs are not mere tools . . . to test psychological explanations; rather . . . themselves the explanations [p.353].

This claim provokes him to a sustained counter-argument, the gist of which is to insist on the difference between simulation and identity, based on the impossibility of separating mind from brain. Intentionality is a biological phenomenon . . . No one would suppose that we could produce milk and sugar by running a computer simulation of the formal sequences in lactation and photosynthesis, but where the mind is concerned many people are willing to believe in such a miracle . . . [p.372].

In their reflections on Searle, Hofstadter and Dennett say unequivocally "our position is quite opposed to Searle's" (p.373) but they do not proceed to face the issues straight on. By implication rather than direct statement they seem to support the strong claims of AI, though they admit that present technology has not yet established the identity of programmed intelligence and human intelligence. "Minds . . . may come to exist in programmed machines. . ." (p.382).

We shall all have to wait and see. Anybody who wishes to confront the unanswerable will enjoy this book; those who struggle with answerable questions will brush it aside.

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Gentle violence

William H. Press

Violent Phenomena in the Universe. By Jayant V. Narlikar. Pp.218. ISBN 0-19-219160-8. (Oxford University Press: 1982.) £9.95, \$19.95.

THE title of this book makes it seem like some tawdry imitation of Nigel Calder's *Violent Universe*. Perhaps some editor with an eye on sales can be blamed for such a blatancy, which does disservice to an excellent and uncompromising book on modern astrophysical theory and discovery. Not just inapt, the title seems quaintly *passé*: "violence" as a cosmic metaphor seems to have faded with the early 1970s. And who now remembers the "majestic" universe, before it became violent?

The universe through which Narlikar leads us is more rational than violent. Its phenomena can be discovered, explored and understood by careful analysis. Moreover, the facts and their analysis can be laid open to the scrutiny of the non-specialist reader.

Precision and economy of expression characterize the narrative as it takes the reader through prerequisite discussions of Newtonian gravitation (and its successful celestial mechanics) to Einstein's theory of gravitation, and then to quite a sophisticated discussion of the formation of black holes and the laws of black-hole physics. The emphasis then waxes astrophysical for a few chapters: stellar evolution, supernovae, neutron stars. By now the reader is ready for pulsars, binary X-ray sources, accretion disks, X-ray bursters, and then a chapter on active galaxies and quasars. The material is extremely up-to-date.

Finally, there are two chapters on cosmology. The first is a clear (if conventional) summary of the big bang, synthesis of the chemical elements, its observed echo in the cosmic microwave background. The second, unfortunately, is on the author's own alternative theory, one favoured by virtually no other practising cosmologist. The author is absolutely fair in introducing this chapter as a "non-standard" and "minority" view; but one is left wondering why he did not apply the same rigorous standard to this chapter as to the previous ones, and delete it *in toto* (an action which the reader should take on his own).

Several stylistic features set this book apart from the myriad other popular and semi-popular books on "black holes and all that stuff": its richness of factual detail; the implicit sophistication in its presentation of the scientific process, the interplay of observation with theoretical hypothesis; its uncompromisingly literate and *numerate* prose. Algebraic formulae are used freely; the reader is assumed to be comfortable with the use of symbols and numbers to represent physical quantities. Further, a sane, methodical organization pervades the whole book. A typical subchapter begins with a question, such as "Does the X-ray source Cygnus X-1 contain a black hole?". Observational facts are then concisely presented, before the author proceeds to discuss the bearing of each of the facts in turn on the question posed.

Probably too sophisticated for a lowest-common-denominator "popular" audience, this book can be recommended strongly to anyone with at least an undergraduate degree in any scientific subject who wants a precise narrative presentation of the excitement of modern astrophysical discovery — and no danger of being talked-down to! □

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Self-instruction in high energy physics

Maurice Jacob

Basics of Electron-Positron Collisions. By Fernand M. Renard. Pp.238. ISBN 2-86332-010-6. (Editions Frontières: 1981.) FF150, \$30.

WITH the decision to build a Large Electron-Positron storage ring at CERN, the LEP project, a large part of future European activity in high energy physics became invested in the study of electron-positron interactions. Already, a tremendous wealth of results has been collected from the e^+e^- colliding ring machines at Stanford (SLAC) and Hamburg (DESY); the LEP project will further extend our ability to investigate the intriguing phenomena thus revealed.

Professor Renard's book on the basics of electron-positron collisions therefore comes at a particularly appropriate time. Many students and experimentalists wish to familiarize themselves with calculations which, over and over again, crop up in the analysis of such collisions. The book should be a good self-teaching guide for that purpose. The author surveys all the standard cases in great detail, giving both explicit formulae and enough of the intermediate steps for their derivation *ab initio*, while always being meticulous with notation. However, in his gallic approach he has a tendency to start with the most general case, discussing only later simpler particular cases, when the beginner might have found the reverse more helpful. The presentation is nevertheless clear enough to enable the reader to skip the general sections easily and go directly to the specific examples.

Reading this book, and making the effort to derive the many formulae given, should prove a very worthwhile investment of time for the dedicated student with some background in relativistic quantum mechanics. The numerous formulae which it contains will also make it useful to the experimentalist writing a Monte Carlo program to assess the response of a detector to expected reactions. To that extent, the book certainly meets a need.

This said, I should add that the newcomer to the field might feel frustrated at the paucity of figures, when the wealth of experimental results is such that calculated distributions and concepts such as jet structure could have been beautifully illustrated. The surprising absence of graphs or tables is also a serious drawback for the reader wishing to assess which

kinematical configurations are the most appropriate for the observation of any particular process. For that reason it is difficult to recommend the book as a reference text; it is pitched, perhaps, too much at the computational level when one could have wished for a more global phenomenological approach. Also, while the scope is quite exhaustive, some sections should be considered merely as brief introductions to particular areas which have come into bloom only recently; this applies especially to the account of quarkonia and jets.

The book thus falls short of providing a balanced, critical survey of research on electron-positron collisions. It should, however, definitely be considered as a useful tool for the dedicated physicist entering this active and exciting field. □

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Dark reactions

M.C.W. Evans

Photosynthesis. Proceedings of the Fifth International Photosynthesis Congress. Edited by G. Akoyunoglou. Six volumes, pp.5,322 excluding indexes. ISBN 0-86689-012-2. (Balaban International Science Services: 1982.) \$499. Single volumes also available. Distributors ISS, S. Pomerantz, 2242 Mt Carmel Ave., Glenside, PA 19038; ISS, Couwenhoven 62-49, 3703 HN Zeist, The Netherlands.

I MUST start this review with two admissions: first I am an author of a very small part of the books under consideration, and secondly I have not read very much of them. However, probably neither is a serious disability. Almost anyone who might review them is an author, and it is highly unlikely that anyone will ever read the entire work. It has 5,322 pages, plus 69 pages of index repeated in each volume, at least 10 million words, and weighs nearly 9 kg. It contains 650 papers written by 850 authors ranging from Abdourakhmanov, I.A., to Zurrer, H., who achieve authorship of between 1 and 13 papers each.

Enough statistics. The books contain the proceedings of a very successful conference held in Halkidiki, in Greece, late in 1980 (see *Nature* 289, 122; 1981). They appear 18 months after the meeting and the question must be, was their publication necessary or worthwhile? The papers are unreviewed and cover a very wide range of subjects from the biophysical studies of light absorption in the picosecond time range,

Yale University Press have recently re-issued Richard Goldschmidt's *The Material Basis of Evolution*, first published in 1940. The reprint incorporates an introduction by Stephen Jay Gould and, in his words, "commemorates a strong reawakening of interest in Goldschmidt's views among evolutionary biologists". Prices are hbk \$35, £25; pbk \$12.95, £9.

through biochemical work on CO₂ fixation, genetic analysis of chloroplast DNA, to physiological ecology. The wide range of subjects is of course a problem for the organizers of the triennial photosynthesis conference, but on balance it is probably a good thing for researchers at the extremes of photosynthesis research to have the chance to hear each other every so often. However they are unlikely, having listened to the talks, to read them again two years later. This is admitted by the editor, who has packaged the volumes into specialist areas for separate sale: the intention does not, then, seem to be to spread a wider view of the world.

Do the books contain much important information which is not available elsewhere? They certainly provide a total view of photosynthesis since so many people attended and dutifully handed in their manuscripts. Much of the work described is of course first class, but even for the best only an outline is provided in the space allocated. For major laboratories a few touches on the world randomizer overcame copyright problems, while for lesser mortals the opportunity to unload all that important work that the refereed journals didn't understand was not to be missed. It is really very unlikely that these volumes contain anything of major scientific importance which has not been published previously: indeed, the smart conferee knows better than to mention anything not already in press with a "good" journal.

Why then did the organizing committee publish the books, and why did the authors bother to submit their papers? The latter question is perhaps easier to explain. Invited speakers no doubt feel some duty to meet the organizers' requirements, while few of us can resist a "free" publication, although surely everyone who is supposed to be impressed by publication lists automatically discounts proceedings of this type. Why the organizers should want to record the proceedings in full is rather harder to understand. There is surely little money to be had from them and an enormous amount of work must have gone into their production. At the Fourth Congress in this series, held in England, no such book was produced, and I do not believe it was seriously missed. It is to be hoped that the organizers of the Sixth Conference next year will show similar restraint.

Who might buy one or all of these books? Libraries, for student use? — review volumes and textbooks are a far better buy. Research laboratories? — refereed journals offer virtually all the useful information. A souvenir of an excellent conference? — a case of ouzo and a set of pornographic coasters would be more fun. No! These volumes are really only for the man who has everything. □

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Philosopher at the bedside

W.F. Bynum

Medical Thinking: A Historical Preface. By Lester S. King. Pp.336. ISBN 0-691-08297-9. (Princeton University Press: 1982.) \$19.50, £14.50.

BRILLAT-SAVARIN's *Philosopher in the Kitchen* showed long ago that every human activity — even cooking and eating — is enhanced by due philosophical reflection. Lester King's philosopher has moved to the bedside where, *malgré lui*, he juggles philosophical concepts when examining his patient, making a diagnosis or prescribing a course of therapy. In using words such as *sign, symptom, syndrome, health, disease, normal, pathological, cause, and effect*, any doctor engages in language possessing a philosophical dimension. The quality of his medical thinking will be reflected in the precision and consistency with which he employs these and similar words. The purpose of King's book is to sharpen that thinking by the historical and philosophical glosses he provides on this central core of medical concepts. It is, King asserts, the kind of book he wishes he had had when he began medical school more than half a century ago.

King's credentials are impressive. After spending 30 years as a pathologist he became a senior editor of the *Journal of the American Medical Association*, a position which permitted him to devote a good deal of time to medical history. His historical researches have ranged widely, but he is a particular student of seventeenth- and eighteenth-century medicine. Those who have followed his earlier work will have noted its increasing philosophical preoccupation; they will also be familiar with many of the doctors — Daniel Sennert (1572–1637), Thomas Sydenham (1624–1689), Hermann Boerhaave (1668–1738), William Cullen (1710–1790) — who people the present volume. Throughout, there is a mixture of historical and contemporary material, as King seeks to identify certain constant features in the interactions between doctors and their suffering patients.

King's central theme may be baldly stated: there has always been good medical thinking, as well as bad, even though medicine's conceptual framework and the amount and quality of medical information available to a doctor have changed dramatically over time (and particularly in the past century or so). In one of the best chapters of the book, King illustrates this constancy in the midst of change by showing how the diagnosis of pulmonary phthisis evolved from the seventeenth to the late nineteenth century with the gradual abandonment of Greek humoralism, development of the tissue concept, invention of the stethoscope, increased use of microscopes and cell theory, and the emergence of bacteriology. The change in name from phthisis to tuberculosis was

part of this process, but pulmonary phthisis was not simply the old name for tuberculosis. Rather, it was a separate disease, often of course including patients suffering from what we would call tuberculosis, but a disease which must be understood historically, within the intellectual frameworks of earlier times. Thus, Richard Morton (1635–1698) cannot be blamed for describing a different disease from the one which intrigued Robert Koch (1843–1910), nor does the fact that Koch worked with 'our' tuberculosis mean *a priori* that he was a better thinker than Morton. Good medical thinking is reflective, critical, and consistent, but is also historically contingent.

The other chapters of King's book are arranged primarily around those concepts intrinsic to medical diagnosis. He examines the historical relationships between signs and symptoms, between concepts of health and of disease, between the 'clinical entity' and the 'disease entity', between disease classification (nosology) and medical knowledge. He has useful things to say on all these and other topics, but some readers may find that three characteristics limit the book's scope. First, it is an extremely personal statement. Many of his references to the secondary literature are to his own earlier writings, and even when citing other work of direct relevance to his topic — for instance A.R. Feinstein's *Clinical Judgment* (Williams & Wilkins, 1967) or F. Kräupl Taylor's *The Concepts of Illness, Disease and Morbus* (Cambridge University Press, 1979) — he makes little attempt to place his own position within its contemporary context. While this gives King's book a greater internal coherence, it also unnecessarily isolates it.

More important, perhaps, is the relatively narrow perspective of disease which King espouses. He is aware of the cultural relativity of disease categories (Chinese women with bound feet are diseased by Western standards), and of the aesthetic judgements which enter definitions of health (straight teeth are 'healthier' than crooked ones). But King's own concept of disease is essentially lesion-orientated. Thus, there is nothing on the notion of mental disease, or of 'functional' illness, or psychosomatic medicine. These softer edges of contemporary medicine are important dimensions of current debate and deserve explicit treatment in a book which is designed to sharpen medical thinking.

Finally, King's philosophy of history has a surprisingly thin social base. Despite his wise injunctions to us to view historical characters historically, he fails to put Sydenham or Cullen in any concrete social milieu. Even epidemiology gets short shrift, an unfortunate omission in two otherwise perceptive chapters on historical

and contemporary concepts of disease causation. It would seem from King's analysis that medicine has its own internal impetus for change, whereas most historians would place more emphasis on social and professional factors.

These lacunae in King's book by no means negate its value. It is a work which

repays thoughtful reading, not least for the way it laments sloppy medical thinking in any age, our own included.

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Unity in diversity — or how to choose your animal

R.B. Moreton

Membrane Physiology of Invertebrates. Edited by Ronald B. Podesta. Pp.664. ISBN 0-8247-1503-9. (Dekker: 1982.) \$87.50.

IN THE animal kingdom, invertebrates not only outnumber the vertebrates to an unimaginable extent, but also present a fascinating diversity of form and function, combined with a surprising unity of basic mechanisms. To those of us who study a specific tissue in depth this provides an opportunity to pick those animals where the particular tissue is especially large, simple or well-developed. Thus, as every physiologist knows, the squid has a giant axon, the sea-urchin lays eggs, the crayfish has a stretch-receptor, and so on. This theme is developed by the late P.C. Caldwell in the introduction to this book, and it runs through the entire volume despite efforts by some authors to collect more general information: invertebrate physiology is just like that.

Membrane Physiology of Invertebrates is a collection of essays by specialist authors, arranged roughly in order of complexity of the organisms covered. It begins with an outline of our disappointingly sketchy understanding of how *Amoeba* feeds, and a detailed analysis of the electrophysiology of the "slipper microbe" *Paramecium*, whose genetic variations have been exploited to study particular membrane functions in isolation.

A chapter on coelenterates (jellyfish and hydroids) provides interesting examples of specialized intercellular junctions, and of the way in which development, for example the growth of new "heads", is controlled by cellular secretions. Intercellular junctions are still more important in the helminths, dealt with in the next contribution, where whole sheets of cells can be fused to form the body-wall. Annelids are covered in chapters on epithelial transport (osmoregulation, digestion and excretion) and excitable cells. The former presents a mainly qualitative study of "what happens where"; the latter begins with a rather confusing attempt to compare the properties of nerve cells in leech and earthworm with those of other groups, but gets into its stride with quantitative modelling

of the giant axon of *Myxicola*. Crustacea are treated similarly, with a detailed and well-illustrated chapter on the gastrointestinal tract, and a good summary of the diverse types of nerve cells and sensory organs that have been studied, including a brief section on barnacle photoreceptors. A chapter by G.A. Gerencser on molluscan digestion presents mainly the author's own work, but refers to other reviews on osmoregulation and transport across the body-wall. The much larger pool of knowledge on molluscan nerve cells is covered, inevitably rather selectively, by F.E. Dudek, who concentrates on gastropods — perhaps enough has been written about the squid axon for the time being! Dudek does include neuromodulators and neuropeptides, however, usefully integrating this relatively new topic with more conventional ideas on synaptic transmission.

Insects are represented by a contribution on gut physiology by W.R. Harvey, which includes an exposition of the thermodynamics of active transport systems, and by a neat summary of the properties of insect muscle, written by F.M. Ashcroft. There is no chapter on insect nerve cells, perhaps due to lack of space to treat this large subject properly. The book ends, unexpectedly, with a short chapter on the echinoderms, dealing mainly with the interesting electrical and chemical events accompanying fertilization of the egg.

This is not a book for the novice: most of the material is detailed and technical, with a fair amount of theory. Rather, it gives a comprehensive view "over the fence" for the specialist in transport or excitable tissue physiology, or for the researcher seeking to enter a new field. The title is misleading, in that much of the material relates to whole epithelia rather than membranes as such. But as a summary of our current knowledge of epithelial transport and excitable tissue physiology, right across the invertebrate kingdom, the book is about as up-to-date as can be expected given the inevitable publication delays. Certainly, it should be in every library.

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Molecular shapes

M.G.B. Drew

Inorganic Stereochemistry. By David L. Kepert. Pp.227. ISBN 3-540-10716-9; 0-387-10716-9. (Springer-Verlag: 1982.) DM154, \$71.70.

CHEMISTS have always had a fundamental interest in the shapes of molecules and in evaluating the reasons for the adoption of a particular geometry. There are two theoretical approaches to such problems: the minimization of non-bonded repulsions and molecular orbital calculations. In this book, Professor Kepert uses simple repulsion theory to evaluate the various geometries of the metal coordination spheres of inorganic complexes in which one central metal atom is surrounded by 4–12 donor atoms.

The author takes each coordination number in turn and calculates the potential energy surface, allowing for possible combinations of unidentate and multi-dentate ligands. The theoretical results are then compared to the observed structural data provided by X-ray analysis. The enormous amount of structural information is thoroughly assimilated and well organized. There is excellent agreement between theory and experimental data, and in all the book amply demonstrates the validity of the repulsion approach to stereochemistry.

Kepert's work has undoubtedly been a major contribution to our understanding of molecular geometry. However it does not seem to have had much impact on the way in which chemists look at molecular geometry. There are probably two main reasons for this. First, the parameters used for the potential energy curves are often not simple to calculate; structural chemists prefer to analyse geometry on the basis of bond lengths, bond angles and δ angles and find Kepert's parameters unnecessarily complicated. Secondly, he does not compare his results with those obtained by other methods. To take just one example, many people find that δ angles are useful structural parameters but they are not mentioned here. Nor indeed are the results of molecular orbital calculations.

It is this single-mindedness that is both the strength and weakness of the Kepert approach: whilst the book is self-contained, it is not intended to provide a balanced view of current ideas on stereochemistry.

It is certainly useful to have Kepert's work brought together in one volume. However the book follows closely a series of reviews written for *Progress in Inorganic Chemistry* over the past few years, and those who have the previous articles may well find this present volume of limited additional value.

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